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DEPARTAMENTO DE BIOLOGIA VEGETAL

SOURCES AND ROUTES OF TRANSMISSION OF Q FEVER: DETECTION, IDENTIFICATION AND MOLECULAR TYPING OF *COXIELLA BURNETII* IN DOMESTIC AND WILD ANIMALS

Aminata Cumbassá

DISSERTAÇÃO

MESTRADO EM MICROBIOLOGIA APLICADA

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Unidade Estratégica de Produção e Saúde Animal

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MASTER THESIS

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ABSTRACT

Query fever (Q fever) is a worldwide zoonosis, affecting many animal species, including Men. This zoonotic disease is caused by *Coxiella burnetii*, an obligate intracellular, Gram-negative filter-passing (0.3 µm) bacterium. Ticks are considered the natural primary reservoir of *Coxiella burnetii*, responsible for the spread of the infection in wild and domestic animals. Domestic ruminants contaminate the environment by shedding *Coxiella* in milk, feces, urine, saliva, vaginal secretions, placenta and amniotic fluids, being the main source of human infection. In animals, *C. burnetii* can cause abortion, premature birth, dead or delivery of weak offspring. Detection of sources of infection and routes of transmission, are essential for the control of *C. burnetii* spread among animals and transmission from animals to humans. The aim of this work is the molecular detection and characterization of *Coxiella burnetii* in animal samples, to elucidate the population structure of this agent in Portugal, for surveillance and epidemiological purposes.

A nested-touchdown PCR assay (Trans-PCR), targeting the repetitive transposon-like element of *C. burnetii* insertion sequence IS*1111*, was performed on 229 DNA tissues and cloacal swab samples, from domestic and wild animals. 19 of them tested positive for *C. burnetii* (14 in domestic and five in wild animals), revealing a prevalence of infection of 8.3% within this sample panel. All 19 cloacal swabs from vultures tested negative.

Multiple locus variable-number of tandem-repeat analysis (MLVA) was used to genotype the 19 *C. burnetii* positive samples, using a set of six VNTR *loci* (Ms23, Ms24, Ms27, Ms28, Ms33 and Ms34). Seven different completed profiles (M1 to M7) and nine partial profiles were observed. The calculated discriminatory power of MLVA was 0.94 for our sample setting, and the diversity indexes (*D*) of the individual markers ranged between 0.73 and 0.95, being loci Ms33 the most discriminatory one.

UPGMA clustering of the MLVA data grouped the *C. burnetii* samples into eight different clusters, being cluster IV the one that included more MLVA types: M1 and M4.

Clustering of the MLVA genotypes using the minimum spanning tree method (MST) grouped cattle and goat samples from this study in one branch, while two samples from the same sheep were grouped completely apart in another branch. Using only completed profiles, this analysis corroborate the hierarchical UPGMA data, confirming cluster IV as the most representative of our sample setting.

None of our samples clustered with animal or human data reported previously in Portugal, or with reference strains, showing a high diversity among the panel sample.

Keywords: Q fever, *C. Burnetii*, infectious disease, MLVA typing

RESUMO

A febre Q é uma zoonose bacteriana com incidência a nível mundial, que afeta muitas espécies animais, incluindo o Homem. Foi descrita pela primeira vez em 1935, em Queensland, Austrália, devido a um surto de doença febril de origem desconhecida, entre os trabalhadores de um matadouro. Como não havia sido descrita até então, foi denominada “Query fever” que significa “febre desconhecida”.

Esta zoonose é causada por *Coxiella burnetii*, uma bactéria intracelular obrigatória, Gram-negativa. A espécie foi isolada pela primeira vez de uma carraça em 1938, em Nine Mile Creek, EUA. As carraças são consideradas o reservatório primário natural da bactéria e são os responsáveis pela sua transmissão aos animais domésticos (bovinos, ovinos, caprinos, suínos, cães, gatos) e selvagens (pássaros e outros), através das suas fezes e/ou mordidas.

Os animais domésticos, por sua vez, são os principais responsáveis pela transmissão da *C. burnetii* aos humanos, através do leite, fezes, urina, saliva, secreções vaginais (especialmente após o parto), placenta e fluidos amnióticos. As infeções por *C. burnetii* podem resultar em abortos, partos prematuros e morte, causando graves transtornos económicos a nível de produção animal.

A deteção das fontes de infeção e vias de transmissão da bactéria são essenciais para o controlo da sua disseminação entre os animais e da sua transmissão dos animais para os humanos.

Este estudo tem como objetivo a avaliação de métodos de deteção molecular e a caracterização de *C. burnetii* em amostras de tecidos animais (domésticos e selvagens), com o propósito de esclarecer qual a estrutura populacional deste microrganismo em Portugal, para fins epidemiológicos e de vigilância.

Analisamos um total de 229 amostras de DNA, extraídos de diferentes tecidos de animais domésticos e selvagens, através da técnica de Trans-PCR, que utiliza dois pares de iniciadores (Trans1/Trans2 e Trans3/Trans4), desenhados para amplificar a região repetitiva tipo-transposónica na sequência de inserção IS*1111* existente no genoma de *C. burnetii*. Trata-se de um PCR “nested” altamente específico e sensível, em que o primeiro par de iniciadores (Trans1/Trans2) amplifica uma região de 687 pb, sendo o produto dessa primeira amplificação utilizado como molde na reação com o segundo par de iniciadores (Trans3/Trans4), que amplificam uma região de 243 pb.

Dezanove amostras (14 animais domésticos e 5 sacarrabos selvagens) foram positivas para *C. burnetii*, revelando uma prevalência de 8,3% no painel de amostras testadas. Todas as 19 zaragatoas cloacais de abutres selvagens analisadas foram negativas para o agente.

As amostras positivas foram posteriormente testadas por qRT-PCR, para quantificar o DNA alvo de *C. burnetii* existente em cada uma, através de valores de cycle threshold (C_t). Esses

valores são inversamente proporcionais à quantidade de DNA alvo existente, uma vez que C_t é definido com o sendo o número de ciclos necessários para o sinal fluorescente exceder o limiar de interferências que possam existir na amostra. Quanto mais baixos os valores de C_t, maior a quantidade de DNA na amostra.

Esta medida permitiu-nos ter uma indicação de quais as amostras que melhores resultados dariam na tipificação por "Multiple locus variable-number of tandem-repeats analysis" (MLVA) pois, de acordo com artigos publicados, amostras com valores de C_t >35 são dificilmente tipificáveis. A tipificação molecular por MLVA foi feita com o objectivo de estudar as principais fontes de infeção e vias de transmissão de *Coxiella*. É uma técnica que se baseia nas variações que ocorrem naturalmente no número de sequências de DNA repetidas em "tandem", no genoma das bactérias. Neste estudo, incluímos todas as 19 amostras, independentemente do C_t obtido, tendo conseguido tipificar algumas de C_t mais alto, apesar de outras de baixo C_t não serem tipificáveis, usando um conjunto de seis *loci* VNTR com unidades repetitivas de seis (Ms27, Ms28 e Ms34) ou 7 pares de bases (Ms23, Ms24 e Ms33).

Obtivemos sete perfis completos diferentes, designados de M1 a M7, e nove perfis parciais, revelando esta técnica uma capacidade discriminatória (HGDI) de 0,93. Para cada marcador individual os índices discriminatórios situaram-se entre 0,73 e 0,95, sendo o *locus* Ms33 o mais discriminatório. Os dados obtidos por MLVA foram agrupados pelo método hierárquico com o coeficiente e método de aglomeração UPGMA [BioNumerics 6.6 (Applied Maths, Bélgica)] em sete grupos diferentes, sendo o grupo IV aquele com maior número de tipos MLVA (M1 e M4).

Utilizando apenas perfis completos para todos os *loci*, verificamos com o método do "Minimum spanning tree" (MST) que as amostras de DNA de *C. burnetii* de bovino, ovino e caprino se agrupavam num ramo, enquanto que duas amostras de ovino do mesmo animal ficavam noutro ramo. Esta análise veio corroborar os resultados da análise hierárquica, confirmando o grupo IV como sendo o mais representativo do nosso conjunto de amostras.

Nenhuma das amostras agrupou com outros perfis, de animais e de humanos, previamente publicados em Portugal, ou com estirpes de referência de *C. burnetii* sequenciadas, mostrando uma grande diversidade e exclusividade das amostras por nós analisadas.

Palavras chave: Febre Q, *Coxiella burnetii*, zoonose infeciosa, vias de infeção, abortos, genotipagem, MLVA.

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LIST OF ABBREVIATIONS

ACCM	Acidified citrate cysteine medium
[CR]	Cybergraphic reference
bp	Base pair
CBA/FCUL	Centro de Biologia Ambiental/Faculdade de Ciências de Lisboa
CEVDI/INSA	Centro de Estudos de Vectores e Doenças Infecciosas/Instituto Nacional de Saúde Dr. Ricardo Jorge
CFT	Complemt fixation test
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EP	Estradas de Portugal
FAM	6-carboxyfluorescein
HEX	Hexachlorofluorescein
IFA	Immunofluorescence Antibody Assay
IHC	Immunohistochemistry
INIAV	Instituto Nacional de Investigação Agrária e Veterinária
LCV	Large-cell-variant
LNIV	Laboratório Nacional de Investigação Veterinária
LPS	Lipopolyssacharide
MLVA	Multiple locus variable-number of tandem-repeats analysis
MST	Minimum spanning tree
N/A	Not available
NADP	Nicotinamide adenine dinucleotide phosphate
OIE	Office International des Epizooties
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qRT-PCR	Quantitaitve real-time PCR
RNA	Ribonucleic acid
SCV	Small-cell-variant
TBE	Tris borate EDTA
TD-PCR	Touchdown PCR
T_m	Melting temperature
U	Units
USA	United States of America
UV	Ultra violet
V	Volts
VERO	African green monkey epithelial
WHO	World Health Organization

1. INTRODUCTION

1.1. Q fever

Q fever is an ubiquitous zoonotic bacterial disease, capable of transmission from animals to humans. *Coxiella burnetii* is the causative agent of Q fever in humans and in animals (also referred as coxiellosis in animals). The disease was first described in 1935 in Queensland, Australia, during an outbreak of a febrile illness of unknown origin, among abattoir workers (Porter *et al*., 2011). As the illness was not previously described, it was named “Q” fever (for query fever). Simultaneously, in Rocky Mountain Laboratory in Montana, USA, in an experiment concerning *Dermacentor andersoni* ticks collected at the Nine Mile Creek, it was isolated an infectious agent with properties not identified before. The agent was infectious for guinea pigs, could pass filters, Gram-negative, and had an extracellular and intracellular pleomorphic, rickettsia-like appearance (Davies & Cox, 1938).

Q fever is presently recognized as being worldwide endemic, with the notable exception of New Zealand (Cutler *et al*., 2007), where seroepidemiological studies were negative for the presence of anti-*C*. *burnetii* antibodies in sera collected from the major ruminant population (cattle and sheep). New Zealand was considered to be free from coxiellosis and thus from human Q fever (Maurin and Raoult, 1999).
In a study to estimate the effect of Q fever used as a bioweapon, the WHO estimated that if 50 kg of *C. burnetii* were aerosolized over an urban area with 500,000 inhabitants, there would be 125,000 cases of acute illness, 9,000 cases of chronic Q fever and 150 fatalities (WHO, 1970) (Oyston and Davies, 2011).

Q fever can be a severe public health problem, and awareness of the disease must be promoted worldwide (Porter *et al*., 2011).

1.2. Sources of infection

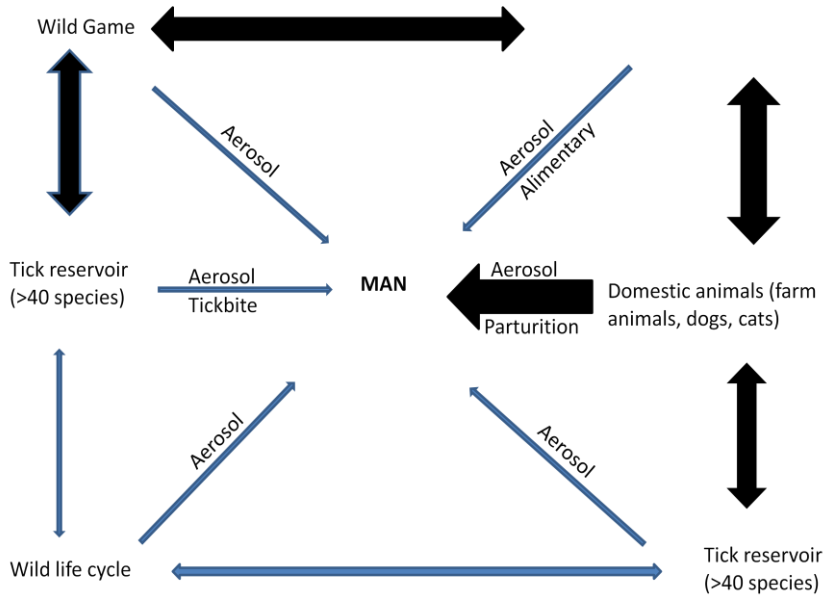

Figure 1 - Transmission cycle of *Coxiella burnetii*. Potential mechanisms of human infection by *C. Burnetii* are shown. Heavy black arrows signify major infection pathways, and thin gray arrows represent minor infection pathways. Humans are considered dead-end hosts and human-to-human infections are very rare (Adapted from (Miller *et al*., 2006).

Ticks are considered to be the natural primary reservoir of *Coxiella burnetii* (Figure 1) and responsible for the spread of the infection in wild animals and for transmission to domestic animals (Kirkan *et al*., 2008). *C. burnetii* can be carried out by more than 40 naturally infected tick species, that transmit the agent both vertically (to their progeny) and horizontally (via bite or in faeces) to wild animals, especially birds and, very rarely, to humans (Miller *et al*., 2006).

Animals are assumed to become infected by inhalation or oral uptake of *C. burnetii* from the environment (Roest *et al*., 2012) and they contaminate the environment by shedding *C. burnetii* in milk, feces, urine, saliva, and very importantly, in vaginal secretions, placenta, amniotic fluids and other products of conception (Porter *et al*., 2011).

Domestic ruminants (cattle, sheep and goats) are considered to be the main reservoir for the pathogen which can infect a large variety of hosts: mammals (humans, ruminants, small rodents, dogs and cats) and also birds, fish, reptiles and arthropods.

1.3. Q fever in domestic and wild animals

C. burnetii infection in animals is generally asymptomatic (Roest *et al*., 2012). However, in cattle, sheep and goats, *C. burnetii* can cause abortion, premature birth, dead or delivery of weak offspring as it localizes in the female reproductive system (Panning *et al*., 2008; Agerholm, 2013).

In symptomatic individuals *C. burnetii* is excreted via faeces, vaginal mucus, milk and birth products.

Little is known on the distribution and incidence of *C. burnetii* in wild life, so the threat that infected animals pose to humans and domestic animals is uncertain (Astobiza *et al*., 2011). However, ticks may play an important role in transmission in the wild, for example between birds (Oyston and Davies, 2011).

1.4. Q fever in humans

Ruminants (sheep, goats and cattle) and pets, namely dogs and cats, are the main sources of human infection (Guatteo *et al*., 2006).

One of the largest outbreaks of Q fever in humans occurred in the Netherlands, between 2007 and 2010, with 4026 human cases reported (Roest *et al*., 2011). It happened during the event "lamb viewing days", an event that occurs every year during lambing season and attracts thousands of people to visit farms. People could watch lambs being born and interact with them. At this time, the risk is greatest because it is when large numbers of highly infectious *Coxiella* strains are shedded. In the year 2007, the event "lamb open house" became "Q-fever open house", due the high number of people that became infected with *C. burnetii* and, consequently, contracted Q fever [CR1].

In humans, Q fever infection usually occur via aerosol (Klee *et al*., 2006), contaminated dust (Panning *et al*., 2008) or ingestion of infected fresh milk and dairy products (Maurin and Raoult 1999).

Q fever is most often asymptomatic in humans (Kirkan *et al*., 2008). When existing, it can manifest as either: acute - a flu-like illness, self-limiting and easily treated with antibiotics; or chronic illness - more severe conditions such as endocarditis, pneumonia and hepatitis, that require prolonged antibiotic therapy (Maurin and Raoult, 1999).

Some studies have shown that men are more affected than women, which may be due to the different employment rates in certain risk professions. "At risk" occupations include, but are not limited to: veterinary personnel, stockyard workers, farmers, shearers, animal transporters and laboratory workers handling potentially infected veterinary samples or visiting abattoirs (Honarmand, 2012).

However, identifying individual farms as primary source for specific clusters of human cases remains a challenge, partly due to limited knowledge of the different *C. burnetii* strains circulating in livestock, the environment and humans (de Bruin *et al*., 2012).

1.5. Characteristics of Coxiella burnetii

C. burnetii is an obligate intracellular, filter-passing (0,3 µm), Gram-negative coccobacillus (Davies and Cox, 1938), belonging to the kingdom of *Bacteria*, phylum of *Proteobacteria*, class of the *Gammaproteobacteria*, order of the *Legionellales*, family of the *Coxiellaceae*, with the genus *Coxiella* and the only species, C. *burnetii*.

C. burnetii is highly infectious and can survive for long periods in the environment (Tilburg *et al*., 2010), due to extracellular spore-like forms (Klee *et al*., 2006), and even a single infective particle can initiate an infection in the animal model (Lorenz *et al*., 1998). Taking into account these facts, this microorganism was classified as a "Category B critical biological agent" by the Centre for Diseases Control and Prevention and is considered a potential weapon for bioterrorism (Porter *et al*., 2011).

The complete genome sequence of *C. burnetii* Nine Mile phase I RSA493 has been obtained and the circular chromosome was found to be 1,995,275 bp in length with a mol % G+C content of 42.6% (Seshadri *et al*., 2003). The Nine Mile strain also possesses a resident plasmid (QpH1) of 37,393 bp. The chromosome contains 29 insertion sequences, 21 being the unique transposon IS*1111*, all of them having more than 99% DNA identity, suggesting a recent introduction into the organism (Miller *et al*., 2006).

C. burnetii strains are known to vary in the type of plasmid carried: QpH1, QpRS, QpDG, QpDV or plasmidless.

The QpH1 plasmid was first obtained from a tick isolate and was also detected in most isolates originating from ticks, domestic animals (cattle, goats, sheep) and acute Q fever patients. The QpRS plasmid was detected in an isolate from an aborted goat and was then found in most isolates from patients with chronic Q fever. The QpDG plasmid was found in only a few isolates from wild rodents (Zhang *et al*. 1998) and the QpDV plasmid has been isolated in a strain from a human case of endocarditis (Porter *et al*., 2011). Plasmidless strains possess plasmid-homologous sequences integrated into the chromosome, implying a critical function for the core plasmid genes (Oyston and Davies, 2011).

The plasmids share significant regions of homology, but also have plasmid-specific sequences which can be used to differentiate them from each other. Generally, plasmids are of little interest for identification of microorganisms because they are not critical for survival and can infect a large variety of microorganisms (Porter *et al*., 2011). However, identification of *Coxiella burnetii*

plasmids may provide important information for the differential diagnosis of Q fever and for epidemiological investigations (Zhang *et al*., 1998).

1.6. Coxiella burnetii pathogenesis

The study of *C. burnetii* pathogenesis has benefited from two recent fundamental advances: improved genetic tools and the ability to grow the bacterium in extracellular media (Van Schaik *et al*., 2013), permitting a better understanding of the species invasion and host cell modulation, including the formation of replication-permissive *Coxiella*-containing vacuoles.

This microorganism has the unique ability to replicate within a large vacuolar compartment inside cells that resembles the acidic environment of a lysosome. Central to its pathogenesis is the delivery of bacterial effectors proteins into the host cell cytosol by a type IVB secretion system. These proteins can interact with and manipulate host factors, thereby leading to creation and maintenance of the vacuole where the bacteria grows (McDonough *et al*., 2013).

Once inhaled or ingested, the extracellular form of *Coxiella burnetii* (or SCV after small-cell-variant) attaches itself to a cell membrane and is internalized into the host cells. After phagolysosomal fusion, the acidity of the newly formed vacuole induces activation of SCV metabolism and its development into LCV, the metabolically active intracellular form of *Coxiella burnetii* (Porter *et al*., 2011).

Two different antigenic forms of *Coxiella burnetii* can be distinguished: phase I and phase II bacterial forms. The difference between these two phases resides in the variation of the surface lipopolysacharide (LPS) as classicaly described for enterobacteria.

Phase variants display different LPS lengths with phase I organisms producing a full-length LPS with O antigen sugars, and phase II organisms producing a truncated LPS without O antigen. This phase variation has been compared to the smooth and rough LPS variation found in *Enterobacteriacae*, with phase II equivalent to the rough LPS phase, and is often, but not always, associated with chromosomal deletion of genes involved in LPS biosynthesis. The phase I form is isolated from infected hosts, but not the phase II form (Oyston and Davies, 2011).

C. burnetii has adapted to the phagolysosomes of eukaryotic cells and is capable of multiplying in the acidic vacuoles, required to activate the metabolism of *C. burnetii* and initiate bacterial replication (Porter *et al*., 2011).

1.7. Isolation and culture of Coxiella burnetii

C. burnetii infection is usually diagnosed by serology and/or PCR detection; however, neither of these methods can determine the viability of the bacterium (Lockhart *et al*., 2012).

Cultivation of *C. burnetii* is very difficult and requires biosafety level 3 conditions, and only a small number of research and public health laboratories and limiting medical practitioners' have access to *C. burnetii* culture for diagnostics.

In Portugal, one of authorised laboratories for isolation and culture of *C. burnetii* is Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge (*CEVDI/INSA*- Águas de Moura), a reference center for Q fever in human.

The microorganism has been first isolated in 1935, from infected ticks in Montana, USA (Davies and Cox 1938) and presently, continuous cell lines such as Vero (African green monkey epithelial cells) and DH82 (canine macrophages) are used for growing this microorganism. Embryonated chicken eggs and animal inoculation have been used for the isolation and growth of large number of cells of *C. burnetii* (Lockhart *et al*., 2012a; Lockhart *et al*., 2012b).

Recently, an axenic medium has been described for *C. burnetii* (Singh *et al*., 2013). It is an acidified citrate cysteine medium (ACCM), which supports host cell-free (axenic) growth of *C. burnetii* over 6 days in a microaerobic environment. A modified version of ACCM called ACCM-2, that supports improved growth of *C. burnetii* in liquid medium and as colonies in agarose plates, has been described (Omsland *et al*., 2011).

1.8. Diagnostic of Q fever: serological methods

At present, serological Enzyme-linked immunosorbent assay (ELISA), direct detection and quantification by PCR should be considered as methods of choice for laboratory diagnosis in animals (Office International des Epizooties - World Organization for Animal Health) [CR2].
The diagnosis should always include a differential investigation of other major abortive agents.
Serological diagnosis of Q fever is usually performed by Immunofluorescent assay (IFA), Complement fixation test (CFT) or ELISA (Tilburg *et al*., 2010).

A skin test method was proposed to investigate the cellular response and to improve the detection of infected cows at herd level. The skin test consists in an intradermal injection of extremely diluted inactivated vaccine (Coxevac, *CEVA-Santé Animale*, Libourne, France). If the animal has previously been infected by Q fever, a nodule of variable size will appear at the site of injection. It is easily applied by rural practitioners (Porter *et al*., 2011).

At INIAV, the serological ELISA diagnostic is done using a "Q-fever Antibody Test Kit" (Idexx, Germany). This kit provides a rapid, simple, sensitive and specific method for detecting antibodies against *Coxiella burnetii* in serum, plasma and milk from ruminants.

In humans, indirect diagnostic methods identify specific humoral or cellular immunity in response to *Coxiella burnetii* infection. These tests are of limited use in the early phase of the disease, as it may take up to two weeks for a detectable immune response to develop (Tilburg *et al*., 2010). Antibodies cannot be detected during the early stage of the infection and it is difficult to discriminate between current and past infection by a test with a single serum sample. Also, serological tests cannot provide the ability to predict whether the patient has acute or chronic disease because they do not detect differences in *C. burnetii* isolates (Zhang *et al*., 1998). Indirect immunofluorescence assay is the diagnostic gold-standard method for serological detection of *C. burnetii*; however, a potential for serological cross-reactivity, with other pathogens, exists (Santos *et al*., 2008).

1.9. Diagnostic of Q fever: molecular methods based on PCR

Detection of shedders of *Coxiella burnetii* is one of the critical points for the control of its spread among animals and transmission from animals to humans. The characteristics of PCR, make it very useful for early diagnosis of infection during the period when antibodies are not yet present. Therefore, specific and sensitive diagnostic PCR systems have been used to detect even small numbers of *coxiellae* (Guatteo *et al*., 2006).

The prerequisite for a diagnostic PCR is a target sequence that is specific for *C. burnetii*, to exclude false positive results with other organisms, and that is conserved and present in all microorganisms, to prevent false negative results (Klee *et al*., 2006). Several PCR-based diagnostic methods, such as conventional PCR, nested PCR, or real-time PCR, have successfully been applied for the direct detection of *C. burnetii* DNA in clinical samples. The sequences targeted by these tests varied from plasmids (QpH1 or QpRS) to chromosomal genes, such as the isocitrate dehydrogenase gene (NADP) or the transposase gene of the *C. burnetii* IS*1111* insertion element. The IS*1111* insertion element is present in approximately 20 copies in the genome of the *C. burnetii* Nine Mile RSA 493 strain and, due to its multicopy nature, it provides a highly sensitive target for detection of *C. burnetii* DNA in clinical samples.

The PCR detects not only infectious agents but non-viable agents as well. It is more sensitive than capture ELISA and is much more rapid and convenient than cell culture, in which at least 6 days of examination is required for diagnostic results (Lorenz *et al*., 1998).

The usefulness of conventional PCR is limited by its inability to quantify the bacteria present. The development of quantitative real-time PCR (qRT-PCR) not only renders PCR a rapid diagnostic tool but also provides quantifiable information. qRT-PCR can be automated and thus can be used in large-scale studies.

In this work a nested PCR assay targeting the repetitive transposon-like element of *C. burnetii* insertion sequence (Trans-PCR), was performed with primers (Trans1/Trans2 and

Trans3/Trans4), based on the referred IS*1111* region. Because it is so highly specific and sensitive it allows the detection of even very few copies of the specific target sequence in different clinical samples (Lorenz *et al*., 1998).

The specificity of PCR is determined by the specificity of the primers. To control for the possibilities of primers binding to more than one locus, nested primers are employed to ensure specificity: two pairs of PCR primers (Trans1/Trans2 and Trans3/Trans4) are used for a single locus. The first pair of primers amplify the locus, and the second pair of primers (nested primers) bind within the first PCR product, producing a second PCR product that will be shorter than the first one [CR3].

A Touchdown PCR (TD-PCR) increases specificity and sensitivity as it employs an initial annealing temperature above the projected melting temperature (T_m) of the primers being used, then progressively transitions to a lower, more permissive annealing temperature over the course of successive cycles. The primer will anneal at the highest temperature, which is least permissive of nonspecific binding. Touchdown increases specificity of the reaction at higher temperatures and increases the efficiency towards the end by lowering the annealing temperature. Any difference in T_m between correct and incorrect annealing will produce an exponential advantage of twofold per cycle (Korbie and Mattick, 2008).

1.10. Molecular typing

Molecular typing of pathogenic microorganisms is mainly used to study transmission routes and to assess sources of infection. Vaccination and use of antibiotics may also interfere on the structure of bacterial populations, and this can be evaluated by comparing molecular typing profiles of members of these populations. Genotypic characterization of *Coxiella burnetii* is a prerequisite for surveillance purposes and for epidemiological investigation of Q fever outbreaks. The information is necessary to evaluate the epidemiological link between the source of the outbreak and human cases, with the final objective of establishing control measures in potential animal hosts involved in the life cycle (Astobiza *et al*., 2012).

The epidemiology of Q fever is complex due to the worldwide distribution, reservoir and vector diversity, and lack of studies defining the dynamic interaction between these factors. In addition *Coxiella* is an agent that could be used as a bioterrorism weapon. Therefore, typing methods that can discriminate strains and be used to trace back infections to their source are of paramount importance. It is also particularly important to compare typing results of different *C. burnetii* strains but culture collections are rare, sparse and not easily transferred due to select agent regulations and biosecurity concerns (Hornstra *et al*., 2011).

According to Astobiza *et al*., (2012) several techniques have been used to genotype and characterize and differentiate *C. burnetii* isolates, based on the sequence analysis of certain

genes such as *com1*, *icd* or *mucZ*. Pulsed field electrophoresis was able to classify *C. burnetii* isolates in different groups; DNA restriction fingerprints and separation by SDS-PAGE differentiated genomic groups.

More recently, two DNA-based methods for typing *C. burnetii* have been described: multiple locus variable-number of tandem-repeats analysis (MLVA) and multispacer sequence typing. These techniques proved to be reliable, reproducible, and with a high discriminatory power. In addition, they do not require previous cultivation of the bacteria and can be implemented directly on clinical and/or environmental samples (Astobiza *et al*., 2012).

Multispacer sequence typing is based on DNA sequence variations in 10 short intergenic regions and can be performed on isolated *C. burnetii* strains or directly on extracted DNA from clinical samples.

Multiple locus variable-number of tandem-repeat analysis (MLVA) utilizes the naturally occurring variation in the number of tandem repeat DNA sequences found in the microbial genome of most bacterial species. The first tandem repeats characterized were in eukaryote genomes. These tandem repeats cover megabases of DNA, and they represent a sufficiently large portion of the genome to be able to produce a “satellite” band on caesium chloride density gradients, so they were called satellite DNA. For this historical reason, the small tandem repeats (in the kilobase range), analyzed by Southern blotting were called minisatellites and later, even smaller structures were called microssatelites (Vergnaud and Pourcel, 2006).

Such tandem repeats may be perfect, but often imperfect repeats containing mutations are encountered (Figure 2).

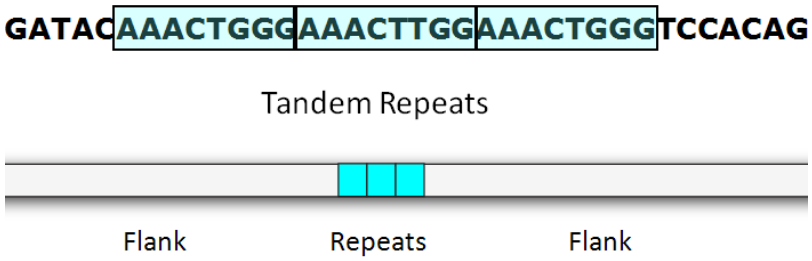

Figure 2 - A tandem repeat *locus* with 8 base pair long tandem repeat units. In the second repeat unit a G has been substituted by a T. The blue boxes indicate the tandem repeats, flanked by DNA regions in gray [CR4].

The number of tandem repeats in a particular *locus* may differ between different strains (Figure 3). Because of this variation, such *loci* are designated as variable number tandem repeat (VNTR) *loci*.

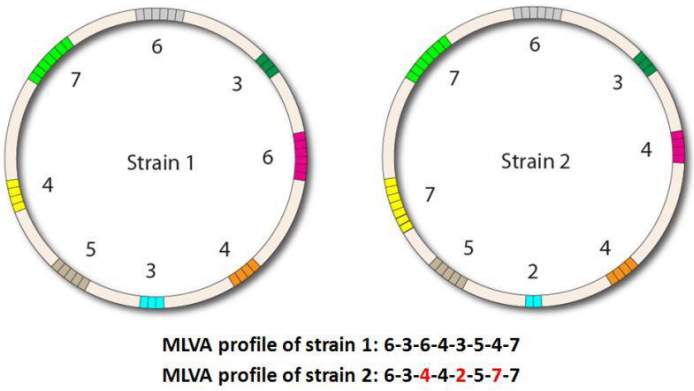

Figure 3 – Example of variation in the number of tandem repeats in multiple VNTR *loci* in two different strains, used to characterize them. The strains differ in three of the eight VNTR *loci,* marked in red [CR4].

Van Belkum *et al*., were the first in 1997 to report a study where they utilize VNTR loci in *Haemophilus* influenza to type this pathogen. In 2000 Keim *et al*., reported a similar approach to type *Bacillus anthracis* and introduced the terminology multiple locus variable-number of tandem-repeat analysis (MLVA). Since then many studies have been performed using MLVA. In 2006, for the first time, two publications (Svraka *et al*., and Arricau-bouvery *et al*.,) reported the development of MLVA for the characterization of *C. burnetii*. Nine unique MLVA types were detected in five laboratory variants and 16 *C. burnetii* isolates, from five different countries, using seven selected MLVA loci ((Svraka *et al*., 2006). These MLVA types were grouped in five different clusters, proving the high discriminatory power of this method. In parallel, (Arricau-Bouvery *et al*., 2006) used 17 loci for MLVA and proposed two panels of MLVA markers: panel 1 composed of 10 minisatellite markers, that could be typed on agarose gel (repeat units longer than 9 bp) and panel 2, composed of 7 microsatellites markers (6 or 7 bp repeat units) with a higher discriminatory power. With these panels, 43 *C. burnetii* isolates could be differentiated in 36 different MLVA types. From there on, several studies were conducted applying this typing methods to both human and animal isolates, and also directly to several *Coxiella* DNA positive samples (Tilburg *et al*., 2010, 2011; Roest *et al*., 2012; Astobiza *et al*., 2012; Hilbert *et al*., 2012; Santos *et al*., 2012).

2. OBJECTIVES

Until two years ago the importance of *Coxiella burnetii* as a pathogenic and zoonotic agent was neglected in animals, mainly ruminants, and other abortive agents, like *Brucella* and *Chlamydia*, were considered more relevant. However, the human outbreaks of Q fever in 2007-2010 in The Netherlands brought a new focus in this disease and incremented studies on reservoirs, sources of infection and routes of transmission of *C. burnetii*.

Therefore, to have a first inside about the animal reservoirs and population structure of *C. burnetii* in Portugal, the main objectives of this study were:

3. To evaluate the presence of *Coxiella burnetii* DNA in a panel of clinical samples (tissues, faeces and swabs) from domestic and wild animals in Portugal;
4. To identify the MLVA genotypes of *Coxiella burnetii* that infect livestock in Portugal;
5. To compare them to other already identified genotypes, by assessing the genetic diversity of the agent;
6. To combine the results, in order to consider the relevance of the infection by *Coxiella burnetii* in animals, as a source and reservoir of Q fever for humans and as the causing agent of economic damages in animal production.

In order to achieve these goals, the following experimental plan was outlined:

- ✓ DNA extraction from sample tissues of domestic ruminants and wild animals;
- ✓ Screening of DNA tissue samples for the presence of *Coxiella burnetii* DNA by Trans-PCR targeting the transposase gene IS*1111* insertion element;
- ✓ Real-Time PCR of *Coxiella burnetii* positive samples in order to quantify the amount of *Coxiella burnetii* DNA present;
- ✓ Molecular typing of the positive cases by plasmid characterization and MLVA (Multiple locus variable-number tandem-repeat analysis);
- ✓ Comparison of the types obtained with others already found in animals and humans.

3. MATERIAL AND METHODS

3.1. Bacterial strains

DNA from *Coxiella burnetii* Nine Mile strain ATCC 616-VR, gently provided by CEVDI/INSA was used in this study. This strain is used for preparation of commercial antigen (Vircell, Spain). The complete genome sequence is available on NCBI/Genbank (accession nr. Y11502).

3.2. Clinical samples

This work was carried out using a total of 229 samples received at the National Institute of Agricultural and Veterinarian Research (INIAV).

One hundred and fifty five samples from production animals, considered to be relevant for detecting *C. burnetii*, were selected based on the animal species and the clinical history, mainly abortions. These samples were submitedto INIAV by veterinarians under their usual practice or by Animal Veterinary Authority (DGAV), under the eradication program for Brucellosis in ruminants. Authorization to use the samples for detection of *Coxiella burnetii* was previously granted.

To evaluate the existence of *Coxiella* reservoirs in the wild and possibility of expel in the environment, 55 DNA samples from wild carnivores tissues and 19 vultures cloacal swabs, were also included. These samples were, respectively, available in the frame of two INIAV collaborations with CBA/FCUL Centro de Biologia Ambiental, Faculdade de Ciências de Lisboa (CBA/FCUL) and Quercos/Centros de Recuperação de Animais Selvagens (CERAS) of Castelo Branco.

Some of the 55 wild carnivores tissues were from road killed animals collected by the road maintenance technicians of ‘‘EP- Estradas de Portugal, S.A.’’ and donated for scientific purposes through a collaboration protocol, entitled “Monitoring of vertebrate mortality caused by road-kill in Portuguese roads" established between CBA/FCUL (Universidade de Lisboa, Centro de Biologia Ambiental, Faculdade de Ciêcias de Lisboa) and ‘‘EP- Estradas de Portugal, S.A.” Other samples were from animals killed in legal hunting sessions (following the Portuguese game legislation) by hunters with valid permits assigned by ‘‘Autoridade Florestal Nacional’’, and totally or partially donated for scientific purposes by the hunting associations/confederations responsible for managing the hunting journeys.

The samples were as follows:

- ✓ 155 tissue samples (liver, spleen, lymph node, mammary gland, uterus, testicles) from domestic animals (cattle, sheep, goat and pigs), with cases of miscarriage, stillbirth and/or suspected brucellosis;
- ✓ 55 DNA samples from wild carnivores (Egyptian mongoose, fox, weasel, genet and badger);

- ✓ 19 DNA samples obtained from cloacal swabs of vultures (*Gyps fulvus*).

3.3. DNA extraction

DNA extraction from organ samples of domestic animals was carried out using the High Pure PCR Template Preparation Kit^{TM} (Roche Diagnostics), according to the manufacturer's instructions. Organ samples were cut into 50 mg sections and mechanically homogenized and disrupted in 200 µl of tissue lyses buffer with beads, using a FastPrep apparatus (Rebolyser), for 40 s at 6 V. Then, 200 µl of lyses buffer containing the disrupted cells were recovered and incubated overnight at 56ºC, with 75 µl of proteinase K (20 mg/ml) (Roche Diagnostics). During the final stage of preparation, the extracted DNA was eluted into 100 µl of elution buffer. Final DNA concentration was determined in a spectrophotometer (NanoDrop 2000 - Thermo Scientific) and DNA stored at -20ºC for further use in PCR assays and typing.

3.4. Trans-PCR assay

The PCR system used was both a nested and a touchdown PCR.

The sensitivity of the Trans-PCR assay for the detection of *C. burnetii* DNA was tested, using serial decimal dilutions (10^{-1} to 10^{-10}), of a known DNA concentration (106.1 ng/µl) from *C. burnetii* Nine Mile strain ATCC 616-VR.

The Trans1/Trans2 and the Trans3/Trans4 primers pairs, were based on the published data sequence of the transposon-like repetitive region of the *C. burnetii* genome. This insertion element is a transposase gene, present in approximately 20 copies (Panning *et al*., 2008) in the genome of *C. burnetii*.

The sequences and direction of polymerization of each primer are detailed in table 1.

Table 1 - Size of C. burnetii fragments amplified by nested PCR and sequences of the oligonucleotides used in the 1st and 2nd reactions. F and R indicate forward and reverse primers, respectively.

	Primer	Primer sequence (5'-3')	Target	Size of the amplicon (bp)	Ref.
1st PCR	Trans 1 Trans 2	F: tatgtatccaccgtagccagtc R: cccaacaacacctccttattc	*C. burnetii* IS*1111* insertion sequence	687	Lorenz *et al*. 1998
2nd PCR	Trans 3 Trans 4	F: gtaacgatgcgcaggcgat R: cccccgcttcgctcgcta		243	

Reactions were performed in a final volume of 25 µl, using 200 µM dNTPs (Promega), 3 mM $MgCl_2$ (Promega), 0.08 µM of each primer (Invitrogen), 0.5 U of GoTaq® DNA polymerase, 1X GoTaq® Flexi buffer (Promega) and 2.5 µl of each DNA dilution. For the second amplification reaction 2.5 µl of the product of the first reaction was used as template. Nine Mile reference strain was used as positive control and double distilled water as negative control.

Amplification was carried out in a Mastercycler gradient thermocycler® (Eppendorf, Germany), under the following conditions (adapted from Lorenz *et al*., 1998): five cycles consisting of denaturation at 94ºC for 30 s, annealing at 66 to 61ºC (the temperature was decreased by 1ºC between consecutive steps – touchdown PCR) for 60 s and extension at 72ºC for 60 s; followed by 40 cycles of denaturation at 94ºC for 30 s, annealing at 61ºC for 30 s and extension at 72ºC for 60 s. Both reactions (1st PCR and 2nd PCR) were performed under these same amplification conditions.

After standardization with the reference strain, field DNA samples were submitted to PCR, using the previously described primers sets (Trans1/Trans2 and Trans3/Trans4), targeting the transposon-like repetitive region of *C. burnetii* IS*1111* insertion element, under the same reaction conditions. *C. burnetii* Nine Mile reference strain DNA was used as the positive control and sterile double distilled water as the negative control.

3.5. Agarose gel electrophoresis

Ten µl of the PCR reactions were analyzed in 1.5% agarose gel electrophoresis in TBE 1X (Tris Borate 40 mM, EDTA 1 mM, pH 8), stained with Ethidium bromide solution (2 mg/ml), and subjected to a constant current of 90 V. Electrophoresis was carried out in 1X TBE buffer, reconstituted from a 5X concentrate TBE solution. Molecular weight DNA markers III and IV from Bioline® were used.

The fragments obtained were analyzed and recorded using a UV transilumination system (BioDoc-It™ Imaging System- UVP).

3.6. Amplification of the constitutive gene of β-actin

After testing all samples by Trans-PCR, all negative ones were tested for β-actin gene (a housekeeping gene, constitutively expressed at a constant level in all living beings), as a way to discard any inhibition of Taq DNA polymerase that may have occurred during both Trans-PCR reactions.

The primers used for this amplification were described by Robinson *et al*. 2007 (Table 2) and they flank a gene segment of about 238 bp.

Table 2 - Oligonucleotides used for β-actin amplification reaction. F and R indicate forward and reerse sequences, respectively.

Primer	Primer sequence (5'-3')	Target	Reference
β-actinBov-F	agcaagcaggagtacgatgagt	β-actin gene	Robinson *et al*. 2007
β-actinBov-R	atccaaccgactgctgtca		

The amplification reaction was performed in a final volume of 25 µl, with 200 µM dNTPs, 3 mM $MgCl_2$, 0.08 µM of each primer, 0.5 U of GoTaq Flexi DNA polymerase (Promega) and 1X of the respective buffer, and 2.5 µl of DNA sample. As negative control, double distilled water was used and, as positive control, DNA extracted from bovine liver.

The reaction was carried out in a Mastercycler gradient thermocycler (Eppendorf, Germany), according to the following conditions: denaturation at 95ºC for 10 min, followed by 35 cycles of 95ºC for 60 s, 50ºC for 40 s and 72ºC for 30 s, with a final extension step of 72ºC for 10 min.

From each PCR reaction, 10 µl of DNA were analyzed by agarose gel electrophoresis 1.5% in TBE 1X (Tris-Borate 40 mM, EDTA 1 mM, pH 8), stained with Gel Red (Biotium) or ethidium bromide solution (1 mg/ml), and subjected to a constant current of 90 V.

The fragment sizes were visualized under UV transilumination (BioDoc-It™ Imaging System) and identified using molecular DNA markers III and VI from Bioline®.

3.7. Purification and sequencing of the PCR amplicons

The resulting final products (amplicons) from Trans-PCR were purified using the QIAquick PCR Purification Kit (Qiagen), according to the manufacturer's instructions. This purification procedure was carried out with the aim of further sequencing of the amplicons, to confirm that they correspond to *C. burnetii* DNA. The purification procedure is schematized in Figure 4.

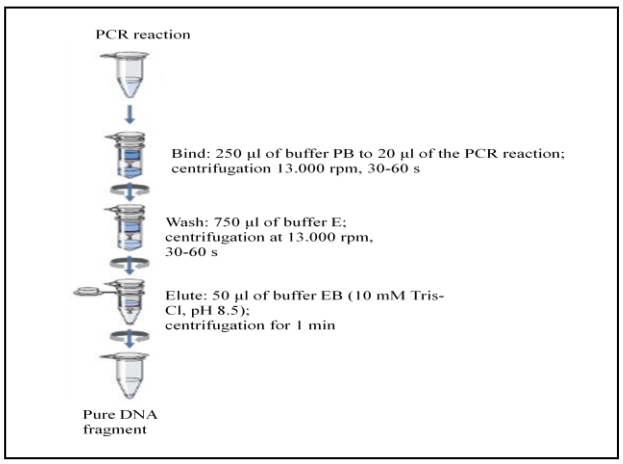

Figure 4 – DNA purification procedure scheme (adapted from [CR5]).

Purified amplicons sequenced in the Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA) in an automated sequencer 3330 XL - Genetic Analyser of 16 capillaries (Applied Biosystems®), using the two internal primers Trans3 and Trans4. The resulting sequences were analysed with "Chromas Lite" version 2.1 (2012) Technelysium Pty Ltd (South Brisbane, Queensland, Australia).

3.8. Quantitative Real-time PCR (qRT-PCR)

A qReal-Time PCR (qRT-PCR) assay was performed in all Trans-PCR positive samples, with the aim of quantifying the amount of *C. burnetii* DNA present in each sample and, thereby, determine whether it was possible to proceed to MLVA typing.

In fact, it has been shown (Santos *et al*. 2012) that MLVA typing of *Coxiella* strains can only be fully achieved on samples with a C_t values within the PCR detection limit. In general, C_t <29 values are strong positive reactions indicative of abundant target DNA in the sample; C_t between 30-37 are positive reactions indicative of moderate amounts of target DNA; C_t of 38-40 are weak reactions indicative of minimal amounts of target DNA [CR6].

A standard calibration curve, with serial dilutions of the reference strain Nine Mile: 10^{-1} to 10^{-6} was designed. Reaction conditions were as follows: 20 µl of total volume, consisting of 10 µl of supermix 1X (Bio-Rad) containing dNTPs, $MgCl_2$, reaction buffer and DNA polymerase, 0.4 µM of each primer (forward and reverse), 0.2 µM of probe and 5 µl of DNA sample. Primers and probe sequences are listed in Table 3.

Table 3 - Oligonucleotides and probe sequences used in the Real-time PCR for quantifying *C. burnetii* DNA load. F and R indicate forward and reverse sequences, respectively.

Primer	Primer sequence (5'-3')
IS*1111aF*	catcacattgccgcgtttac
IS*1111aR*	ggttggtccctcgacaacat
Probe *IS1111a*	aatccccaacaacacctccttattcccac

Amplification was carried out in aC1000 Thermal cycler (Bio-Rad®), conditions were as follows (adapted from Roest *et al*., 2011): one cycle consisting of denaturation/activation at 95ºC for 120 s, followed by 40 cycles of denaturation at 95ºC for 10 s, and annealing at 60ºC for 30 s. Results (on C_t values) were generated with CFX Manager Software (Bio-Rad, Germany).

3.9. Plasmid-specific PCR to identify plasmids QpH1 and QpRS

Differentiation of *C. burnetii* plasmid types offers an important tool for epidemiological investigations; therefore, a plasmid-specific PCR assay was performed with the aim of determining which plasmid exists on the reference strain (Nine Mile ATCC 616-VR) used in this study, as well as, in all samples that were positive for *C. burnetii* in the Trans-PCR assay.

Specific primers (CB5-CB6 and QpRS1-QpRS2) used for this reaction are listed on table 4.

CB5-CB6 primers pair was drawn from a particular QpH1 plasmid gene (*cbhE'*) and they amplify a region of about 977 bp. Primers QpRS1-QpRS2 were designed from a single QpRS plasmid gene (*cbbE'*), amplifying a region of about 693 bp (Zhang *et al*. 1998).

Table 4 - Oligonucleotides used for plasmid-specific PCR. F and R indicate forward and reverse primers, respectively.

Target	Primer	Sequence (5'-3')	Gene detected	Amplicon size (bp)	Ref.
Plasmid QpH1	CB5 CB6	F: ataatgagattagaacaaccaaga R: tctttcttgttcattttctgagtc	*cbhE'*	977	Zhang, *et al*. 1998
Plasmid QpRS	QpRS1 QpRS2	F: ctcgtacccaaagactatgaatatatcc R: aacaccgatcaatgcgactagccc	*cbbE'*	693	

Amplification was performed in a final volume of 25 µl, using 200 mM dNTPs (Promega), 3 mM $MgCl_2$ (Promega), 62.5 µM of each primer (Eurofins MWG), 0.5 U of GoTaq ® DNA polymerase (Promega), 1X GoTaq ® Flexi buffer (Promega) and 2.5 µl of DNA sample.

Reaction conditions for both plasmids (QpH1 and QpRS) are listed on the table below.

Table 5 - PCR reaction conditions for plasmids QpH1 and QpRS

Stage	Temp. (ºC)	Time (s)	Cycles
Initial denaturation	94	120	1
Denaturation	94	120	
Annealing	56	60	35
Extension	72	120	
Final extension	72	60	1

After the PCR reaction, an agarose gel (1.5%) electrophoresis was performed under the same conditions described previously. The fragment sizes were identified under UV transilumination as above.

3.10. Multiple locus variable-number tandem-repeat analysis (MLVA) typing

In MLVA, the analysis of variation in the number of repeats in a set of VNTR *loci* is achieved by performing PCR, using primers flanking that region, and subsequent sizing of the PCR products on agarose gels, capillary systems or automated DNA sequencers.

MLVA typing was performed using three hexanucleotide repeat markers (Ms27, Ms28 and Ms34) and 3 heptanucleotide repeat markers (Ms23, Ms24 and Ms33) as target *loci*, directly in 19 DNA clinical samples (14 DNA samples from domestic ruminants and five DNA samples from wild carnivores), that had been positive for *C. burnetii* by Trans-PCR analysis.

The PCR reaction was performed in a Mastercycler gradient thermocycler® (Eppendorf, Germany), in a total volume of 25 µl containing 1 U of GoTaq® DNA polymerase (Promega), 1X GoTaq ® Flexi buffer, 4 mM $MgCl_2$, 0.2 mM of dNTPs, 0.5 µM of amplification primers (table 6) and 5 µl of DNA template. DNA from the Nine Mile strain (ATCC 616-VR) was used as a positive control and sterile bidistilled water as a negative control.

Amplification cycles were adapted from Roest *et al*. 2011 and consisted of an initial step of denaturation/activation for 2 min at 95ºC, followed by 40 cycles of denaturation for 30 s at 95ºC, annealing for 30 s at 58ºC, elongation for 30 s at 72ºC, followed by a final extension step for 5 min at 72ºC.

Table 6 – Characteristics of each l*oci* and set of primers used in MLVA typing

Locus	Alias	Size (bp) /sequence of repeat motive*	Consensus motive**	Forward primer	Reverse primer	Amplicon size (bp)/Nr.of repeats***	Reference
Ms23	Cox6; Cbu0197	7 / GAGGGCA 7 / GAAGACA 7 / GAGGACA	GA**R**G**R**CA	HEX-CGCMTAGCGACACAACCAC	GACGGGCTAAATTACACCTGCT	133/9	Tilburg *et al*. 2012
Ms24	Cox4; CbuO259	7 / GACGGAA 7 / GACAGAA 7 /GACAGAG	GAC**R**GA**R**	FAM-TGGAGGGACTCCGATTAAAA	GCCACACAACTCTGTTTTCAG	261/27	Tilburg *et al*. 2012
Ms33	Cbu1435	7 / CTGTCTT	--	NED-TCGCGTAGCGACACAACC	GTAGCCCGTATGACGCGAAC	104/4	Tilburg, *et al*. 2012
Ms27	Cox2; CbuO838	6 /TGAAGA	--	HEX-TCTTTATTTCAGGCCGGAGT	GAACGACTCATTGAACACACG	89/4	Klaassen *et al*. 2009
Ms28	Cox5; CbuO839	6 / TAAGAA 6 / TAAGGA	TAAG**R**A	NED-AGCAAAGAAATGTGAGGATCG	GCCAAAGGGATATTTTTGTCCTTC	111/6	Klaassen *et al.* 2009
Ms34	Cox1; Cbu1471	6 / GAAAAG	--	FAM-TTCTTCGGTGAGTTGCTGTG	GCAATGACTATCAGCGACTCGAA	101/5	Klaassen *et al.* 2009

*****Obtained by *in silico* analysis of genomic sequence of Nine Mile strain RSA493 (NCBI/GenBank accession number NC_002971) and according to Tilburg *et al*. (2012) and Klaassen *et al*. (2009). In *loci* Ms23, Ms24 and Ms28 the sequence of the motive is not conserved in all repeats, differing in one (Ms28) or two nucleotides (Ms23 and Ms24). **A consensus repeat motive is proposed for these *loci*, based on the International Union of Pure and Applied Chemistry (IUPAC), where R can be any purine base (A or G); ***in reference strain Nine Mile RSA493 using the above primers.

3.10.1. Capillary analysis of MLVA PCR products

VNTR PCR products can be separated and analysed in a DNA sequencer, to evaluate its size. Fluorescently labeled primers are used and each VNTR *locus* has its own fluorescent label indicated by different colors. The high resolution separation of the sequencer enables accurate and high throughput analysis (≤ 2 base resolution).

Each 19 PCR products, with different fluorescent dyes were mixed with 12 µl of Hi-Di formamide (Applied Biosystems) and 0.2 µl of Rox 500 Size Standard (Applied Biosystems). After denaturation for 5 min at 90ºC, the PCR products were separated in an ABI PRISM 310 Genetic Analyser (Applied Biosystems, Germany) with a 36 cm array by using POP7 polymer.

Electropherograms were analysed using ABI PRISM GeneScan 3.7 (Applied Biosystems). DNA from the Nine Mile strain (ATCC 616-VR) was used as reference.

3.10.2. Agarose gel eletrophoresis analysis of MLVA PCR products

The MLVA results were also analyzed by agarose gel electrophoresis, as agarose gels can be easily read manually, revealing the size variation of the PCR products (Vergnaud and Pourcel, 2006).

The PCR amplication products (10 µl) were analyzed by agarose gel electrophoresis 3% in TBE 1X (Tris Borate 40 mM, EDTA 1 mM, pH 8), stained with Ethidium bromide solution (2 mg/ml) and subjected to a constant current of 160 V for 3 h.

The fragment sizes were visualized under UV transilumination (UV transiluminator Bio Doc-It™ Imaging System) and identified using a 20 bp molecular DNA marker from Bio-Rad®.

3.10.3. Assessing the number of tandem repeats

The PCR product size was used to calculate the number of repeat units in each *locus.* The flanking regions, analyzed *in silico* including the hybridization sites of the primers used, were subtracted from the PCR product size, resulting in the net size of the repeat region. Small inaccuracies in sizing may occur but mostly do not prevent the assessment of the number of repeats.

The accuracy of the sizing was determined by comparing sequence data from the reference strain, with the obtained fragment size from the capillary electrophoresis and agarose gel electrophoresis and corrected, if necessary. The number of repeats for each locus was determined on the basis of the published and corrected annotation of the various *loci* (Table 8) [CR7].

3.10.4. Analysis and clustering of MLVA data

The calculated number of repeats of the VNTR *loci* was combined into a string, referred to as the MLVA profile. Each unique MLVA profile was given a MLVA type designation (M1 to M7). The copy number for each locus was managed as a character dataset using BioNumerics version 6.6 (Applied Maths, Belgium). Cluster analysis was based on the categorical coefficient and unweighted pair group method using arithmetic averages (UPGMA).

MLVA typing tools and databases with MLVA profiles of several organisms, including links to tandem repeats which have already been investigated, were accessed over the internet [CR7; CR8; CR9; CR10; CR11; CR12 and CR13].

3.10.5. Determination of the discriminatory power (D) of the MLVA typing

The Discriminatory Power (*D*) or Hunter & Gaston Discriminatory Index (HGDI) is determined by the number of types defined by the test method and the relative frequencies of these types. It is based on the probability that two strains chosen at random from a population of unrelated strains will be distinguished by a chosen typing method (Hunter and Gaston, 1988):

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j(x_j - 1)$$

Where *xj*(*xj*-1) is the number of strains in the population which are indistinguishable from the *j*th strain and *N* is the number of strains in the population. Each strain in turn is compared with all other strains in the population to determine how many other strains are indistinguishable from it to give *xj*(*xj*-1) (Hunter 1990).

The discriminating power of the typing method and the genetic diversity of each locus were determined using the Hunter & Gaston diversity index (*D*) via an online tool available at the website [CR13].

We applied this equation to determine the discriminating power of the typing method and for each individual *locus*. Results are presented on the final summary table 8.

4. RESULTS AND DISCUSSION

4.1 Determination of the detection limit of the Trans-PCR assay

Primer pairs Trans1/Trans2 and Trans3/Trans4 were used to detect *C. burnetii* DNA (106.1 ng/µl) from the Nine Mile reference strain ATCC 616-VR, in different dilutions.

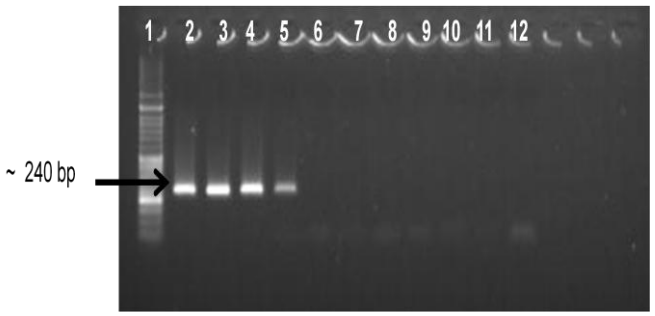

Figure 5 - Sensitivity of Trans-PCR for the detection of *C. burnetii* Nine Mile strain ATCC 616-VR DNA (specific 240 bp amplicon). Lane 1: DNA molecular marker (100-bp); lanes 2-11: different dilutions (10^{-1}-10^{-10}) of the template DNA; lane 12: negative control (bidistilled sterile water).

The analytical sensitivity of the Trans-PCR was found to be 1 fg/µl (Figure 5 – lane 5). This result is compatible to that obtained (later on) with qRT-PCR, confirming the suitability of the Trans-PCR assay for routine diagnostic of *C. burnetii*.

4.2. Detection of C. burnetii DNA in clinical samples using Trans-PCR assay

Initially, we used 239 macerated, already processed samples of different organs (liver, spleen, uterus, mammary gland, testicles) from cattle, sheep and goats, received in INIAV – Bacteriology, Lisboa, for bacteriological diagnosis of *Brucella* spp.

Since all of them tested negative for *C. burnetii*, we decided to change strategy and use other samples where the extraction of DNA was done directly from the tissues and not from the macerates. These samples were sent by Bacteriology, Vairão.

We believe that the negative results we had achieved in the first set of samples, were not due to the lack of DNA from *C. burnetii*, but due to the dilution of samples because of maceration, even though it was a highly sensitive and specific PCR.

In the image below (Figure 6) are presented two examples of *C. burnetii* Trans-PCR positive results, in tissue samples (liver and spleen) obtained from an ovine aborted fetus, with the specific 240 bp amplified fragment.

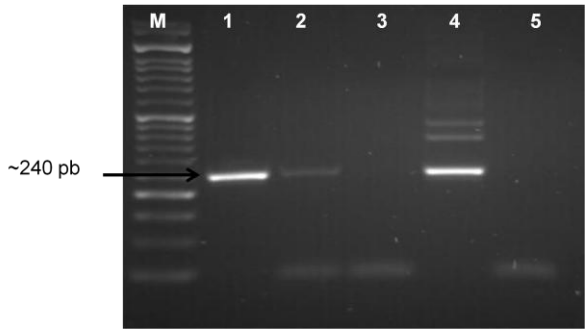

Figure 6 – Results of Trans-PCR in samples from a sheep fetus - analysed in agarose gel (1.5%). M – Molecular weight DNA ladder VI (Bioline); samples 1 to 3:1– liver; 2 – spleen; 3 – gastric content; 4 – Positive control (Nine Mile strain ATCC 616-VR); 5 – Negative control (sterile bidestilated water).

Small ruminants (sheep and goats) are referred as the main source of infection by *C. burnetii*. The results show that much more cases of infection by *C. burnetii* may occur, in these species and cause miscarriages. Such cases remain undetected, since the focus is on other, more well-known abortive microbial species.

From other 229 samples further tested from different sources, 19 samples (14 from domestic ruminants and five from wild carnivores, namely Egyptian mongoose), were positive for *C. burnetii* in nested PCR (table 7). Based on PCR results applied to our panel of samples, a total prevalence of *Coxiella burnetii* infection in farm animals and wild life was estimated to be 8.3%. Goats were the main carriers of *C. burnetii* infection (4.7%), followed by E. mongooses (2.2%). Cattle and sheep had a low prevalence of infection (0.43%). None of the 19 vulture (*Gyps fulvus*) cloacal swabs were positive for *C. burnetii* DNA. In a study about occurrence of *C. burnetii* in wildlife in Northern Spain (Astobiza *et al*., 2011), 11% of *Gyps fulvus* samples were positive but tissues of dead animals were used, instead of swabs, like we did.

The information about the absence of other abortive microbial species (e.g., *Brucella*), in the clinical samples analysed in this study, is relevant as it allows to conclude that the symptoms (abortion) observed might have been caused by infection with *Coxiella burnetii* (Table 7). however, veterinary practitioners are not yet alerted for this possibility.

There are in the literature reports (Georgiev, *et al*., 2012) that highlight the gaps in knowledge about the seroprevalence of *C. burnetii* or clinical prevalence of infection, both in farm animals and in humans. Estimating the Q fever incidence in farm animals is difficult, due to the non-specific nature of the disease and the multifactorial nature of abortion. Further, it is uncommon for detailed veterinary investigations to occur, including efforts towards laboratory confirmation of the causative agent, after single abortions in a herd or flock. The disease is well recognized among the veterinary community in Netherlands, Germany and Bulgaria, where it is a notifiable disease since 2008, in opposite to France and other European countries like Portugal. The presence of a natural reservoir in wildlife, as we detected in mongooses, with eventual spill-over to farm animals, is considered a pre-requisite for endemicity of Q fever in a geographic region.

4.3. Amplification of the β-actin constitutive gene

For this procedure, we applied primers targeting bovine β-actin, also with the aim to see to what extent these primers would recognize sequences in sheep and goats.

In the Figure 7 below, we present an example of positive cases of β-actin PCR amplification, where we can observe the expected amplification product for all species although with some inespecificity. This may be due to changes in the specific sequences but, these changes did not prevent annealing under the conditions used for amplification.

Since one of our purposes was also to verify if the Taq-polymerase was not inhibited, the amplification confirms the full functioning of the enzyme. This allowed us to confirm the negatives as true results, as they were not due to inhibition of the enzyme.

The reaction was not performed in duplex (with Trans-PCR primers), because the amplified fragment of β-actin is about the same size (238 bp) as the fragment obtained by Trans-PCR (243 bp). In duplex, these two fragments would co-migrate, not being possible to conclude to which of them (β-actin or the transposase gene of *C. burnetii*) the band corresponds to.

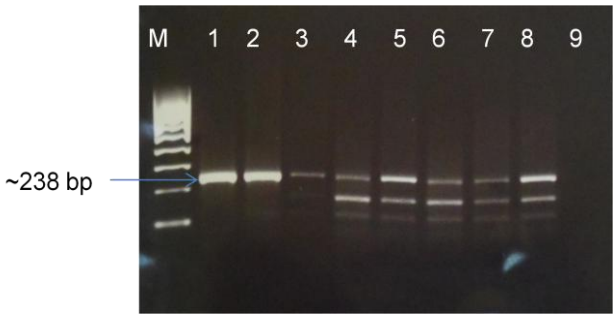

Figure 7 – Image of an agarose gel (1.5%) electrophoresis corresponding to the amplification of β-actin gene from DNA samples of small ruminants. M – Molecular weight DNA ladder III (Bioline); 1 – bovine liver; 2 – bovine spleen, 3 – goat ganglium; 4 – goat mammary gland; 5 – goat uterus; 6 – goat liver; 7 – goat spleen; 8 – sheep uterus; 9 - Negative control (sterile bidestilated water).

4.4. Amplicon sequencing

The analysis of sequencing results, by comparison with the sequence of the transposase gene (IS*1111*) of *C. burnetii* Nine Mile strain in the EMBL/Genbank database (accession nr. M80806) confirmed that the amplicons obtained in the Trans-PCR corresponded to *Coxiella burnetii* DNA specific fragment.

4.5. Quantitative Real-time PCR (qRT-PCR)

A quantitative real-time PCR (qRT-PCR) assay was performed in all 19 previously described (nested PCR) positive samples. A standard calibration curve was obtained using triplicate samples for each dilution of the reference strain, Nine Mile ATCC 616-VR (Figure 8).

C_t (cycle threshold) values for each sample are presented on table 8.

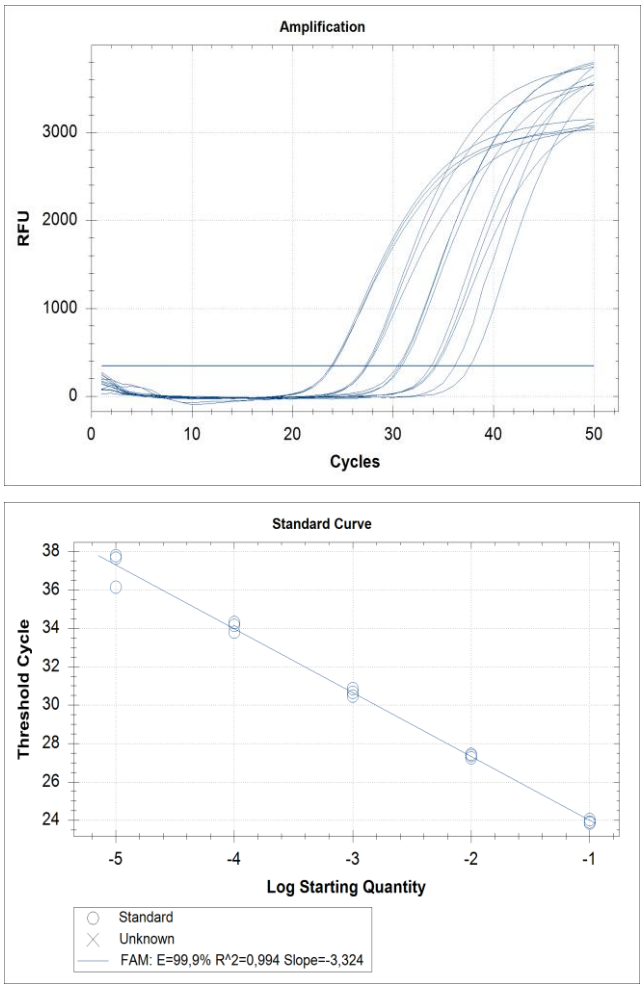

Figure 8 - Amplification graphic and standard curve obtained with the *C. burnetii* reference strain Nine Mile ATCC 616-VR.

From the standard curve above, we verified that with C_t values above 34-35, the correct detection and quantification of DNA was not possible.

The detection limit of this system corresponds to a DNA concentration of 1 fg/µl (C_t= 34), exactly the same as the one obtained for the Trans-PCR assay. This data explains Santos *et al*. (2012) observation that only C_t values below 35 give MLVA satisfactory results.

Despite that, all samples from this study, independently of their C_t value, were included for typing. Seven samples (698, 705, 4121, 4732/1, 33783/1, B7, B9) held C_t values of 46.62, 48.45, 39.37, 39.01, 35.58, 35.96 and 35.72, respectively. No C_t values were obtained for samples 776 and 23301/2 (Table 8).

Only samples 4325/1 and 4325/2 from the same goat, liver and lung, gave positive results in both Trans1/Trans2 and Trans3/Trans4 and it was the lowest C_t value found in ruminants (25.5 and 27.08, respectively). Accordingly all the six MLVA *loci* were amplified. Other low C_t values were three mongoose tissues (B31, B40 and B57) where, probably due to DNA degradation, not all MLVA *loci* were amplified (Table 8).

4.6. Plasmid-specific PCR to identify plasmids QpH1 and QpRS

Investigation of samples with specific primers for plasmid sequences gave the following results: reference strain Nine Mile ATCC 616-VR gave positive result only with primers QpRS1/QpRS2 in dilution $106.1x10^{-1}$ ng/µl corresponding to a DNA concentration of 1 mg/µl, amplifying a product of about 693 bp, the exact size expected for this primers pair, which corresponds to plasmid QpRS (figure 9).

Coxiella burnetii strain RSA493 is a Nine Mile phase I isolate which contains a small plasmid QpH1 (complete genome available on EMBL/Genbank database accession nr. NC_004704). In our study, we used DNA from a Nine Mile (ATCC 616-VR) reference strain, utilized as antigen in the preparation of serological tests by a Spanish enterprise (Vircell) and no amplification was observed with the primers pairs CB5-CB6 (QpH1). This indicates that the DNA sample of the reference strain used does not contain QpH1 plasmid but is, surprisingly, a QpRS plasmid-containing strain. Phylogenetic analysis of 173 C*. burnetii* isolates from 21 different countries, based on multispacer sequence typing and plasmid sequence type (Glazunova *et al*., 2005) revealed that in some isolates QpH1 plasmid evolved to QpRS plasmid. However, this assumption is mere speculation in the case of Nine Mile DNA used in this study and no information was available, in ATCC site, about the characteristics of this strain.

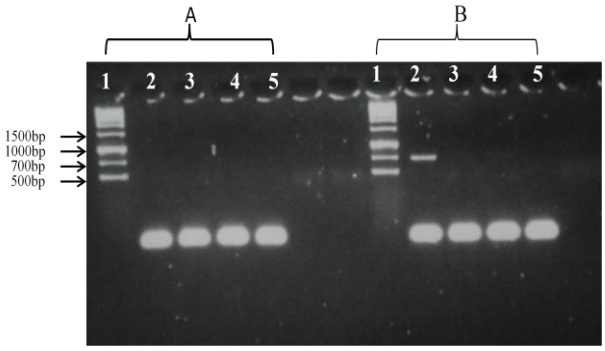

Figure 9 - Identification of the QpH1 (A) and QpRS (B) plasmids in the reference strain ATCC 616-VR, by PCR, with primer pairs CB5-CB6 and QpRS1-QpRS2, respectively. **A**: Lane 1 – molecular size marker (500-bp DNA ladder); lanes 2 to 4, three serial dilutions of the reference strain Nine Mile: $106.1x10^{-1}$ ng/µl, $106.1x10^{-2}$ ng/µl and $106.1x10^{-3}$ ng/µl, respectively; lane 5 – negative control (sterile bidestilated water). **B**: Lane 1 – molecular size marker (500 bp DNA ladder); lanes 2 to 4, three serial dilutions of the reference strain Nine Mile: $106.1x10^{-1}$ ng/µl, $106.1x10^{-2}$ ng/µl and $106.1x10^{-3}$ ng/µl, respectively; lane 5 – negative control (sterile bidestilated water).

With respect to the samples, none of them gave amplification with any of the primers (CB5-CB6 for plasmid QpH1 and QpRS1-QpRS2 for plasmid QpRS) and so, it seems that the samples had plasmidless *C. burnetii*.

Another explanation for these results might be that, since we are not working with pure DNA from *C. burnetii* isolates but with a complex DNA mixture that includes DNA from the host, the extracromosomal DNA from plasmids, due to its small dimension, may be overwhelmed by other DNA, remaining undetected. With the conventional PCR system used, the detection of plasmid DNA might only be possible in pure DNA from *C. burnetii*, unless a more sensitive PCR is applied, like nested PCR used by Zhang *et al*., (1998) in human sera and by Arricau-Bouvery *et al*., (2006), in domestic animals tissues samples and human blood. Therefore, based on these results, we could not say with confidence whether the *C. burnetii* DNA detected was plasmidless or not.

4.7. MLVA typing

The number of repeats in each of six markers (Ms23-Ms24-Ms27-Ms28-Ms33-Ms34) was determined by capillary analysis and agarose gel electrophoresis. A correspondence between size of amplicons, with the primers described and number of repeats in each *locus* was done by extrapolation, with the sizes obtained in DNA from the Nine Mile reference strain (appendix - table 1). According to published data, the genotype of the Nine Mile RSA493 strain is 9-27-4-6-4-5 for markers Ms23-Ms24-Ms27-Ms28-Ms33-Ms34, respectively [CR6], which, with the primers used, correspond to a certain amplicon size (Table 8). Since not all repeats of the same *locus* have exactly the same sequence, a consensus motive was created for *loci* Ms23, Ms24 and Ms28 (Table 6). We also adopted the consensus number of repeats for *locus* Ms33 in

reference strain Nine Mile RSA493, according to Arricau-Bouvery *et al*., (2006), since other interpretation can be made (8 instead of 4 repeats), depending on the criteria used for choosing the repeat motive. Samples 4325/1 and 4325/2 and 33783/1 and 33783/2, from goat and sheep, respectively, correspond to different organs from the same animal and presented the same MLVA profile as should be expected and plausible, showing the repeatability of the method.

From 19 typed *C. burnetii* DNA positive samples, seven completed different profiles were obtained (M1 to M7) and nine partial profiles. The calculated discriminatory power of MLVA was 0.94 for our sample setting, using the above six loci. Therefore, MLVA is a valuable tool for epidemiological studies. The diversity indexes (*D*) of the individual markers were 0.85, 0.91, 0.73, 0.76, 0.95 and 0.93 for Ms23, Ms24, Ms27, Ms28, Ms33 and Ms34, respectively. All loci show a high discriminatory power being loci Ms33 the most discriminatory one (Table 8).

Results of the UPGMA clustering of the MLVA data showed the genetic relationships among the MLVA profiles grouping the *C. burnetii* samples into eight clusters. In the UPGMA cluster analysis we used all 19 MLVA profiles from this study, independently if they were complete or partial, and included previous data (Santos *et al*., 2012) from a human patient (1658) and from four goats [390, 685 (lung and liver), 747].

MLVA profiles of *C. burnetii* sequenced isolates (*C. burnetii* CbuK Q154, *C. burnetii* CbuG Q212 and *C. burnetii* RSA331) retrieved from data bank (accession numbers CP001020, CP001019, CP000890, respectively), and Tilbourg *et al*., (2011) were also included (Figure 10).

This analysis revealed the existence of eight clusters: I, II, III, IV, V, VI, VII and VIII, within the samples tested in this study, using a cut-off value of 40% (Figure 10). Cluster I is a single member cluster, consisting of one cattle sample from north of Portugal; Cluster II consists of samples from one goat and one sheep from the north of Portugal and includes MLVA partial profiles; cluster III is also a single member cluster from one goat from the north, with M2 MLVA type; cluster IV includes samples from four goats from the north region, with M1 and M4 MLVA types and two partial profiles; cluster V includes M7 MLVA type, corresponding to three mongooses from the north and south of Portugal; cluster VI includes M3 MLVA type from two goat samples from the same animal, also from north of Portugal; cluster VII includes M6 MLVA type from two mongooses from the centre of Portugal; cluster VIII includes the MLVA type M5 corresponding to two sheep samples from the same animal (Figures 10 and 11).

None of our samples clustered with animal or human data reported previously in Portugal, or with reference strains. The great genotype diversity of the tested samples was the most outstanding conclusion of this analysis.

Clustering of the MLVA genotypes using the minimum spanning tree method – MST (Figure 11), gave a simpler representation of the genetic relations of the *C. burnetii* DNA samples. Only completed MLVA profiles were included. The lines represent relations among the MLVA types. The short solid lines represent a relation of five identical *loci* of the six and the longer solid line of less than five of the six. The dotted lines represent a very loose relationship (two, one or none of the six loci are identical). The dimension of the dots represents the number of samples that cluster together. From the cluster formed by samples 390, 747 and 685 (previous data from Santos *et al.*, 2012) three branches were formed. In the right branch clusters most of the cattle and goat samples from this study, while two sheep samples from the same animal are completely apart grouping in the left branch. Both UPGMA and MST methods gave comparable results. Roughly, eight major clusters were apparent in UPGMA analysis and, since in MST only completed MLVA profiles were included, only six clusters are formed. This MLVA typing can be refined if DNA from *C. burnetii* isolates is used. For this, clinical *C. burnetii* positive samples have been sent to INSARJ-CEVDI to accomplish the isolation of the agent.

None of the profiles we found, have been previously identified in animal or human clinical samples from several European countries, being all of them described for the first time in this work. The genetic diversity of MLVA profiles found is in agreement with results obtained with *C. burnetii* positive domestic ruminant samples from northern Spain (Astobiza *et al*., 2012).

The diversity of *C. burnetii* in Portugal, contrasts with the genotypic identity, found in The Netherlands, between *C. burnetii* from humans (Tilburg, *et al*., 2011) and goats (Roest, *et al*., 2011). In a scenario of a Q fever outbreak, as the one that occurred in The Netherlands, a clonal spread of *C. burnetii* seems to occur, what is not the case in Portugal or in Spain.

Table 7 – *Coxiella burnetii* positive samples from this study, with reference to the isolation of *Brucella* spp.

Sample ID	Host	Clinical sample	Geograph. Location	Year of collection	Symptoms/ cause of death	Isolation of *Brucella* spp.	Detection of *C. Burnetii* (Trans-PCR)	
							t1/t2	t3/t4
534	Cattle	Spleen/liver	North	2012	Abortion	Negative	Negative	Positive
698	Sheep	Spleen/liver	North	2012	Abortion	Negative	Negative	Positive
705	Goat	Spleen/liver	North	2012	Abortion	Negative	Negative	Positive
776	Goat	Spleen/liver	North	2012	Abortion	Negative	Negative	Positive
815	Goat	Spleen/liver	North	2012	Abortion	Negative	Negative	Positive
4121	Goat	Liver	North	2012	Abortion	Negative	Negative	Positive
4325/1	Goat	Liver	North	2012	Abortion	Negative	Positive	Positive
4325/2	Goat	Lung	North	2012	Abortion	Negative	Positive	Positive
4732/1	Goat	Liver	North	2012	Abortion	Negative	Negative	Positive
4732/2	Goat	Lung	North	2012	Abortion	Negative	Negative	Positive
23301/1	Goat	Liver	North	2012	Abortion	Negative	Negative	Positive
23301/2	Goat	Lung	North	2012	Abortion	Negative	Negative	Positive
33783/1	Sheep	Liver	Center	2012	Abortion	Negative	Negative	Positive
33783/2	Sheep	Spleen	Center	2012	Abortion	Negative	Negative	Positive
B7	Egyptian mongoose	Spleen	Center	2011	Predator density control (PDC)	NA	Negative	Positive
B9	Egyptian mongoose	Spleen	Center	2011	Predator density control (PDC)	NA	Negative	Positive
B31	Egyptian mongoose	Spleen	North	2011	Predator density control (PDC)	NA	Negative	Positive
B40	Egyptian mongoose	Spleen	South	2011	Predator density control (PDC)	NA	Negative	Positive
B57	Egyptian mongoose	Spleen	Center	2011	Predator density control (PDC)	NA	Negative	Positive

Table 8 – Summary table. Clinical samples, host, geographical location, year of collection, Ct values and obtained MLVA genotypes. The discriminatory power index (*D*) values for the typing method and for each locus are presented in the two last rows.

Sample ID	Host	Geographical location	Year of collection	C_t value	Nr. of repeats						MLVA type	Source
					Ms23	Ms24	Ms27	Ms28	Ms33	Ms34		
534	cattle	North	2012	34.17	5	-	-	3	-	20	Partial	this study
698	sheep	North	2012	46.62	7	6	2	-	72	28	Partial	this study
705	goat	North	2012	48.45	35	-	2	2	-	-	Partial	this study
776	goat	north	2012	NA	6	12	2	8	28	30	M1	this study
815	goat	north	2012	34.02	29	6	2	8	33	29	M2	this study
4121	goat	north	2012	39.37	6	12	2	15	-	8	Partial	this study
4325/1	goat	north	2012	25.51	6	15	23	8	91	7	M3	this study
4325/2	goat	north	2012	27.08	6	15	23	8	91	7	M3	this study
4732/1	goat	north	2012	39.01	6	12	2	16	91	32	M4	this study
4732/2	goat	north	2012	32.02	6	12	2	-	-	32	Partial	this study
23301/1	goat	north	2012	28.66	-	1	-	-	-	-	Partial	this study
23301/2	goat	north	2012	NA	-	-	-	-	-	-	-	this study
33783/1	Sheep	center	2012	35.58	57	4	5	1	76	10	M5	this study
33783/2	sheep	center	2012	34.22	57	4	5	1	76	10	M5	this study
B7	mongoose	center	2011	35.96	49	37	14	-	7	5	Partial	this study
B9	mongoose	center	2011	35.72	49	38	14	21	37	20	M6	this study
B31	mongoose	north	2011	20	14	17	14	8	43	20	M7	this study
B40	mongoose	south	2011	24.27	-	24	14	8	8	-	Partial	this study
B57	mongoose	center	2011	19.05	14	15	14	8	12	-	Partial	this study
390	goat	center	2010	16.97	1	11	2	3	2	3	S	Santos *et al.*, 2012
685	goat	Spain	2010	21.62	1	11	2	3	2	3	S	Santos *et al*., 2012
685	goat	spain	2010	22.55	1	11	2	3	2	3	S	Santos *et al*., 2012
animal isolate 747	goat	center	2007	12.14	1	11	2	3	2	3	S	Santos *et al*., 2012
patient 1658	human	center	2007	15.82	3	9	4	5	2	2	T	Santos *et al*., 2012

C. burnetii Dugway	NA	ND	5	4	4	3	3	Tilburg *et al*., 2012
C. burnetii RSA 331	NA	4	7	3	3	-1[b]	3	Tilburg *et al*., 2012
C. burnetii RSA493	22	9	27	4	6	4	5	this study; [CR7]
C. burnetii CbuG Q212	NA	ND	8	3	4	2	2	Tilburg *et al*., 2012
C. burnetii CbuK Q154	NA	ND	9	4	5	2	2	Tilburg *et al*., 2012
D (n=9)					0.94			
D (n=19)		0.85	0.91	0.73	0.76	0.95	0.93	

The number of repeats in each marker was determined by extrapolation using the sizes of the obtained fragments relative to those obtained using DNA from the Nine Mile strain RSA 493.

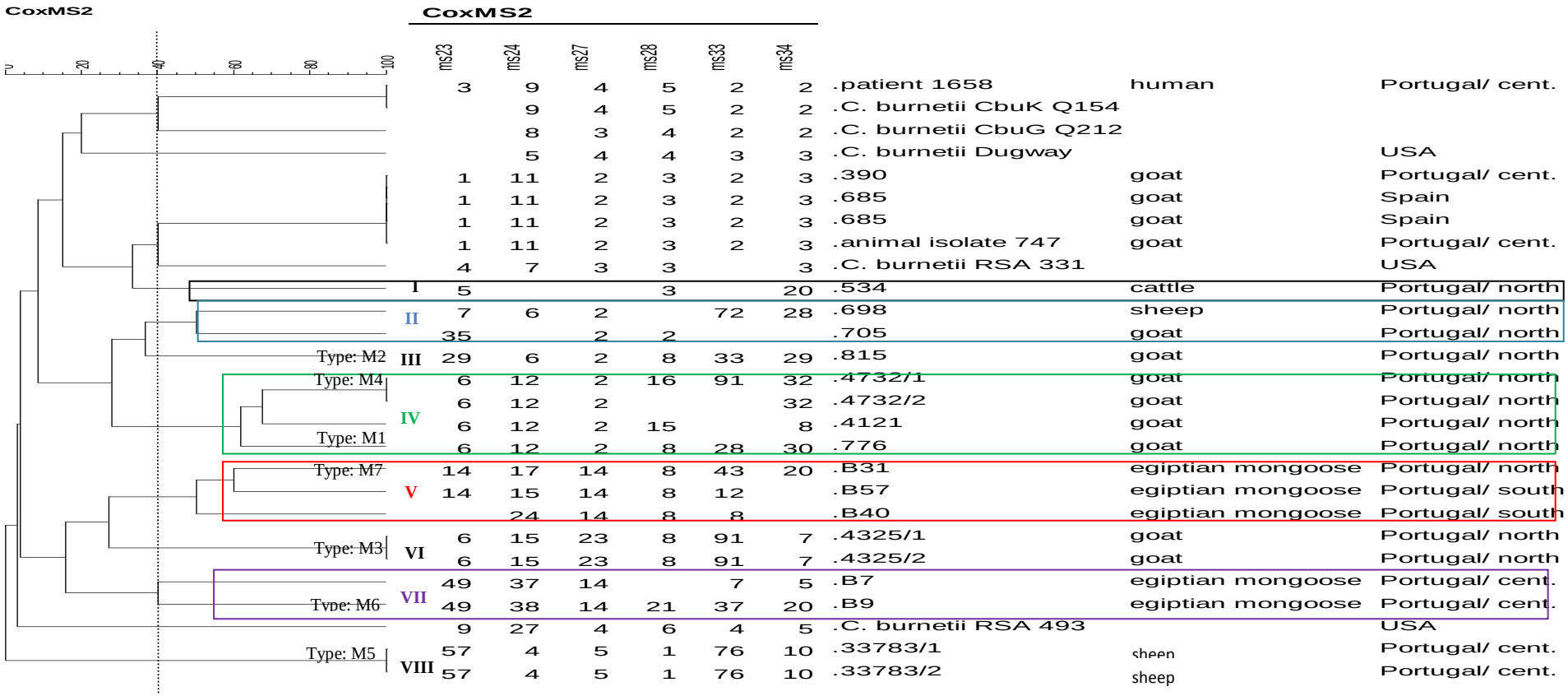

Figure 10 - Dendrogram constructed with 17 samples from this study (534, 698, 705, 776, 815, 4121, 4325/1, 4325/2, 4732/1, 4732/2, 33783/1, 33783/2, B7, B9, B31, B40 and B57);four animal (390, 685 lung, 685 liver, 747) and one human data (patient 1658) (Santos *et al*., 2012) and four additional sequenced *C. burnetii* strains (Dugway, RSA331, CbuG Q212, CbuK Q154) (Tilburg *et al*., 2012), on the basis of six multiple locus variable-number of tandem-repeats analysis (MLVA). Repeats per locus are shown. Samples (23301/1 and 23301/2) with only one or no genotype were excluded. Open spots indicate missing values.

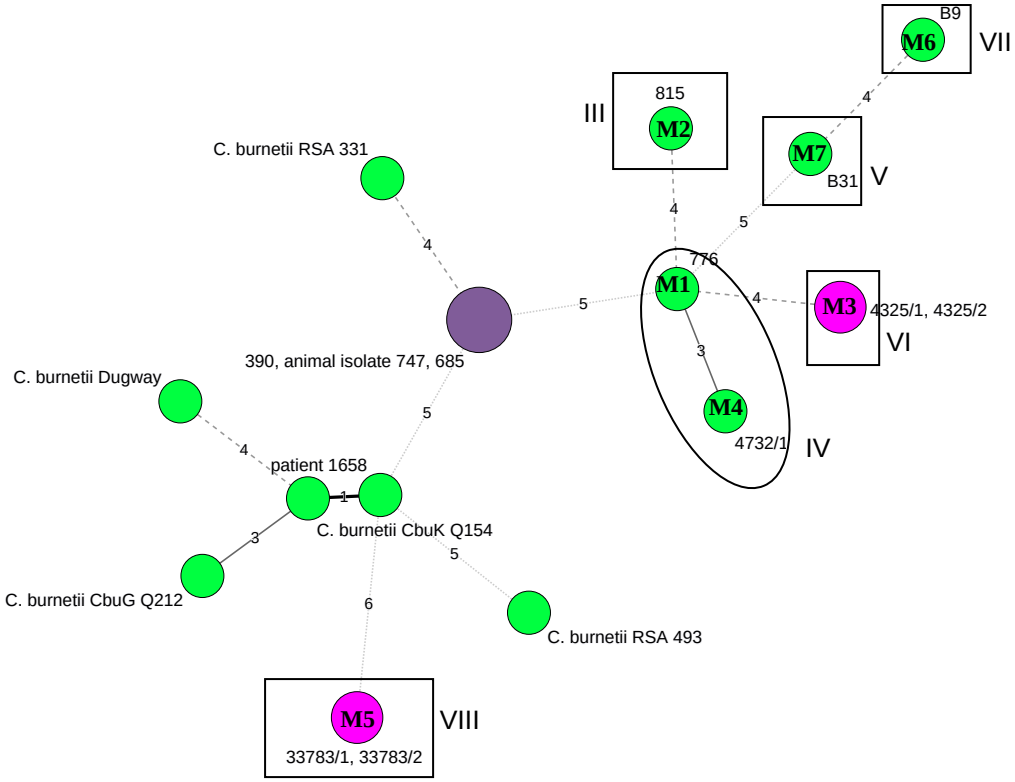

Figure 11 - Minimum spanning tree, showing a representation of the differences between the MLVA genotypes found in nine (776, 815, 4325/1, 4325/2, 4732/1, 33783/1, 33783/2, B9 and B31) animal clinical samples and the reference strain Nine Mile RSA 493. Only full MLVA genotypes were included in this analysis. Results from previous studies (Santos *et al*., 2012) and from published results of *C. burnetii* reference strain (Tilburg *et al*., 2011) and *in silico* analysis of database sequences, were also included. Numbers in the lines represent the number of different loci between samples. MLVA types are indicated within the circles and corresponding clusters sideways. Circles in green represent 1 sample; circles in pink represent 2 samples and purple circles represent three samples.

5. CONCLUSIONS

In view of the results obtained in this study, the following conclusions can be drawn:

1. Abortions in *C. burnetii*-infected domestic ruminants, mainly goats, seem to be the most important excretion route of this microorganism in Portugal, confirming other authors' results in different countries.

2. Considering the small number of samples, wild animals, as Egyptian mongoose, can, surprisingly play an important role as reservoirs and/or spill over of *C. burnetii* in Portugal.

3. The population structure of *C. burnetii* in Portugal is very heterogeneous, as verified using our panel of samples and the 6 MLVA *loci* (Ms23, Ms24, Ms27, Ms28, Ms33 and Ms34).

4. The lack of standardization of the MLVA method, assigning of *loci* and interpretation of data, is an important constrain to compare results from studies of different authors.

5. From this work we realize that the capacity for diagnosis and awareness of animal Q fever must be implemented in a routine base. This will increase familiarity with the presentation of Q fever in humans, resulting in more rapid diagnosis of clinical cases.

6. However, epidemiological studies on *C. burnetii* infection in animals and Q fever in humans need to be interpreted with care, given differences in the underlying epidemiological conditions and the study designs used (sample size, target groups, and study purpose).

7. A limited number of *C. burnetii* positive samples (19) were available to this study, and more samples, and also isolates, will have to be tested in the future to better characterize the population structure of this microorganism in Portugal. A plan for animal samples collection, suspected of *C. burnetii* infection will be designed to cover all the country.

8. To our knowledge, this constitutes the first large study on *C. burnetii* animal infection in Portugal and the data obtained is extremely relevant for the knowledge of Q fever in this part of the World. A very recent study (Anastácio *et al*., 2013), concerning the seroprevalence to *C. burnetii* in sheep and goats, from the central region of Portugal revealed an herd prevalence of 32,6%, while individual prevalence was about 9,6%. Positive results were significantly associated with goats, older animals and larger herds.

These informations will be taken into account in the design of a study to detect type and isolate *C. burnetii* in the center of Portugal.

9. A network involving, physicians, veterinarians, epidemiologists and bioinformatics, relying on an “One Health” approach, is surely needed in Portugal.

6. REFERENCES

Agerholm, J. S. (2013). *Coxiella burnetii* associated reproductive disorders in domestic animals - a critical review. *Acta Veterinaria Scandinavia*, 1-11.

Amano, K.-I., & Williams, J. C. (1984). Chemical and immunological characterization of lipopolysacharides from phase I and phase II *Coxiella burnetii*. *Journal of Bacteriology*, 994-1002.

Anastácio, S., Tavares, N., Carolino, N., Sidi-Boumedine, K., & da Silva, G.J. (2013). Serological evidence of exposure to *Coxiella burnetii* in sheep and goats in central Portugal. *Veterinary Microbiology*, 6313-6319.

Arricau-Bouvery, N., Hauck, Y., Bejaoui, A., Frangoulidis, D., Bodier, C. C., Souriau, A., Meyer, H., Neubauer, H., Rodoloakis, A., & Vergnaud, G. (2006). Molecular characterization of *Coxiella burnetii* isolates by infrequent restriction site-PCR and MLVA typing. *BMC Microbiology* .

Astobiza, I., Barral, M., Ruiz-Fons, F., Barandika, J. F., Gerrikagoitia, X., Hurtado, A., & García-Pérez, A.L. (2011). Molecular investigation of the occurence of *Coxiella burnetii* in wild life and ticks in an endemic area. *Veterinary Microbiology*, 190-194.

Astobiza, I., Tilburg, J. J., Piñero, A., Hurtado, A., García-Perez, A. L., Nabuurs-franssen, M. H., Marriegje, H., & Klaassen, H. W. (2012). Genotyping of *Coxiella burnetii* from domestic ruminants in northern Spain. *BMC Veterinary Research*, 1-8.

Clemente, L., Barahona, M. J., Andrade, M., & Botelho, A. (2009). Diagnosis by PCR of *Coxiella burnetii* in aborted fetuses of domestic ruminants in Portugal. *Veterinary Record*, 1-3.

Clemente, L., Fernandes, T. L., Barahona, M. J., Bernardino, R., & Botelho, A. (2008). Confirmation by PCR of *Coxiella burnetii* infection in animals at a zoo in Lisbon, Portugal. *Veterinary Record*, 221-222.

Cutler, S. J., Bouzid, M., & Cutler, R. R. (2007). Q fever. *Journal of Infection*, 313-318.

Davies, G. E., & Cox, H. R. (1938, December 30). A filter-passing infectious agent isolated from ticks. *Public Health Reports* , 2259-2282.

de Bruin, A., van Alphen, P. T., van der Plaats, R. Q., de Heer, L. N., Reusken, C. B., van Rotterdam, B. J., & Janse, I. (2012). Molecular typing of *Coxiella burnetii* from animal and environmental matrices during Q fever epidemics in the Netherlands. *BMC Veterinary Research*, 1-9.

Fard, S. N., & Khalili, M. (2011). PCR-detection of *Coxiella burnetii* in ticks collected from sheep and goats in southeast Iran. *Iran J Arthropod-Borne Dis*, 1-6.

Glazunova, O., Roux, V., Freylikman, O., Sekeyova, Z., Fournous, G., Tyczka, J., Tokarevich, N., Kovacova, E., Marrie, T.J., & Raoult, D. (2005). *Coxiella burnetii* genotyping. *Emerging Infectious Diseases*, 1211-1217.

Guatteo, R., Beaudeau, F., Berri, M., Rodolakis, A., Joly, A., & Seegers, H. (2006). Shedding routes off *Coxiella burnetii* in dairy cows: omplications for detection and control. *Vet. Res.*, 827-833.

Hirai, A., Nakama, A., Chiba, T., & Kai, A. (2012). Development of a method for detecting *Coxiella burnetii* in cheese samples. *The Japanese Society of Veterinary Science, 74* (2), 175-180.

Honarmand, H. (2012). Q fever: an old but still a poorly understood disease. *Interdisciplinary Perspectives on Infectious Diseases* , 1-8.

Hoover, T. A., Vodkin, M. H., & Williams, J. C. (1992). A *Coxiella burnetii* repeated DNA element resembling a bacterial insertion sequence. *Journal of Bacteriology*, 5540-5548.

Hornstra, H. M., Priestley, R. A., Georgia, S. M., Kachur, S., Birdsell, D. N., Hilsabeck, R., Gates, L.T., Samuel, J.E., Heinzen, R.A., Kersh, G.J., Keim, P., Massung, R.F., & Pearson, T. (2011). Rapid typing of *Coxiella burnetii*. *PLOS one*, 1-8.

Hunter, P. R. (1990). Reproducibilit and indices of discriminatory power of microbial typing methods. *Journal of Clinical Microbiology*, 1903-1905.

Hunter, P. R., & Gaston, M. A. (1988). Numerical index of the discriminatory ability of typing systems: an application of Simpson's Index diversity. *Journal of Clinical Microbiology*, 2465-2466.

Kirkan, S., Kaya, O., Tekbiyik, S., & Parin, U. (2008). Detection of *Coxiella burrnetii* in cattle by PCR. *Turk. J. Vet. Anim. Sci.*, 215-220.

Klaassen, C. H., Nabuurs-Franssen, M., Tilburg, J. J., Hamans, M. A., & Horrevorts, A. M. (2009). Multigenotype Q fever outbreak, The Netherlands. *Emerging Infectious Disesas*, 613-614.

Klee, S. R., Tyczka, J., Ellerbrok, H., Franz, T., Linke, S., Baljer, G., & Appel, B. (2006). Highly sensitive real-time PCR for specific detection and quantification of *Coxiella burnetii*. *BMC Microbiology* .

Korbie, D. J., & Mattick, J. S. (2008). Touchdown PCR for increased specificity and sensitivity in PCR amplification. *Nature Protocols*, 1452 - 1456.

Lockhart, M. G., Islam, A., Fenwick, S. G., Graves, S. R., & Stenos, J. (2012). Comparative sensitivity of four different cell lines for the isolation of *Coxiella burnetii*. *FEMS Microbiolofy Letters*, 75-78.

Lockhart, M., Islam, A., Graves, S., Fenwick, S., & Stenos, J. (2012). detecting and measuring small numbers of viable *Coxiella burnetii*. *immunology and Medical Microbiology*, 61-65.

Lorenz, H., Jäger, C., Willems, H., & Baljer, G. (1998). PCR detection of *Coxiella burnetii* from different clinical specimens, especially bovine milk, on the basis of DNA preparation with a silica matrix. *American society for Microbiology*, 4234-4237.

Massung, R., Cutler, S., & Franqoulidis, D. (2012). Molecular typing of *Coxiella burnetii* (Q fever). *Advances in Experimental Medicine and Biology,* 381-396.

Maurin, M., & Raoult, D. (1999). Q fever. *Clinical Microbiology Reviews*, 518-553.

McDonough, J. A., Newton, H. J., Klum, S., Swiss, R., Agaisse, H., & Roy, C. R. (2013). Host pathways important for *Coxiella burnetii* infection revealed by genome-wide RNA interference screen. *American Society for Microbiology*, 1-13.

Miller, J., Shaw, E., & Thompson, H. (2006). *Coxiella burnetii*, Q fever and Bioterrorism. In B. Anderson, H. Friedman, & M. Bendinelli, *Microorganisms and Bioterrorism* (pp. 181-208). New York: Springer - Science + business Media, Inc.

Omsland, A., Beare, P. A., Hill, J., Cockrell, D. C., Howe, D., Hansen, B., Samuel, J.E., & Heinzen, R.A. (2011). Isolation from animal tissue and genetic transformation of *Coxiella burnetii* are facilitated by an improved axenic growth medium. *Applied and Environmental Microbiology*, 3720-3725.

Oyston, P., & Davies, C. (2011). Q fever: the neglected biothreat agent. *Journal of medical Microbiology*, 9-21.

Panning, M., Kilwinski, J., Greiner-Fischer, S., Peters, M., Kramme, S., Frangoulidis, D., Meyer, H., Henning, K., & Drosten, C. (2008). High throughput detection of *Coxiella burnetii* by real-time PCR with internal control system and automated DNA preparation. *BMC Microbiology*, 8-77.

Porter, S. R., Czaplicki, G., Mainil, J., Guattéo, R., & Saegerman, C. (2011). Q Fever: Current state of knowledge and perspectives of research of a neglected zoonosis. *International Journal of Microbiology*, 1-22.

Raoult, D., Vestris, G., & Enea, M. (1990). Isolation of 16 strains of *Coxiella burnetii* from patients by using a sensitive centrifugation cell culture system and establishment of the strains in HEL cells. *Journal of Clinical Microbiology*, 2482-2484.

Roest, H. I., Ruuls, R. C., Tilburg, J. J., Nabuurs-Franssen, M. H., Klaassen, C. H., Vellema, P., Van Den Brom , R., Dercksen, D., Wouda, W., Spierenburg, M.A.H., Van Der Spek, A.N., Buijs, R., De Boer, A.G., Willemsen, P.T.J., & Van Zijderveld, F.G. (2011). Molecular epidemiology of *Coxiella burnetii* from ruminants in Q fever outbreak, the Netherlands. *Emerging Infectious Diseases*, 668-675.

Roest, H., Tilburg, J., van der Hoek, W., Vellema, P., van Zijderveld, F., Klaassen, C., Raoult, D. (2011). The Q fever epidemic in The Netherlands: history, onset, response and reflection. *Epidemiol. Infect*., 1-12.

Roest, H.-J., Van Gelderen, B., Dinkla, A., Frangoulidis, D., Van Zijderveld, F., Rebel, J., & Van Keulen, L. (2012). Q fever in pregnant goats: pathogenesis and excretion of *Coxiella burnetii*. *PLOS ONE*, 1-14.

Rousset, E., Berri, M., Durant, B., Dufour, P., Prigent, M., Delcroix, T., Touratier, A., & Rodolakis, A. (2009). *Coxiella burnetii* shedding routes and antibody response after outbreaks of Q fever-induced abortion in dairy goat herds. *Applied and Environmental Microbiology*, 428-433.

Santana, M. F., Silva, J. C., Batista, A. D., Ribeiro, L. E., da Silva, G. F., de Araújo, E. F., & de Queiroz, M.V. (2012). Abundance, distribution and potential impact of transposable elements in the genome of *Mycosphaerella fijiensis*. *BMC Genomics* .

Santos, A. S., Bacellar, F., & França, A. (2007). Febre Q: revisão de conceitos. *Revista da Sociedade Portuguesa de Medicina Interna*, 90-99.

Santos, A. S., de Sousa, R., Alves, F., Proença, P., Núncio, M. S., Dumler, J. S., & Bacellar, F. (2008). Isolation of *Coxiella burnetii* from the blood of a patient with positive Anaplasma phagocytophilum serological results. *European Society of Clinical Microbiology and Infectious Diseases*, 1-2.

Santos, A. S., Tilburg, J. J., Botelho, A., Barahona, M. J., Núncio, M. S., Nabuurs-Franssen, M. H., & Klaassen, C. H. W. (2012). Genotypic diversity of clinical *Coxiella burnetii* isolates from Portugal based on MST and MLVA typing. *International Journal of Medical Microbiology*, 253-256.

Seshadri, R., Paulsen, I. T., Eisen, J. A., Read, T. D., Nelson, K. E., Nelson, W. C., Ward. N. L., Tettelin, H., Davidsen, T. M., Beanan, M. J., Deboy, R. T., Daugherty, S. C., Brinkac, L. M., Madupu, R., Dodson, R. J., Khouri, H. M., Lee, K. H., .; Carty, H. A., Scanlan, D., Heinzen, R. A., Thompson, H. A., Samuel, J. E., Fraser, C. M., & Heidelberg, J. F. (2003). Complete genome sequence of the Q-fever pathogen *Coxiella burnetii*. *Proceedings of the National Academy of Sciences*, 5455-5460.

Singh, S., Eldin, C., Kowalczewska, M., & Raoult, D. (2013). Axenic culture of fastidious and intracellular bacteria. *Trends in Microbiology*, 92-99.

Svraka, S., Toman, R., Skultety, L., Slaba, K., & Homan, W. L. (2006). Establishment of a genotyping scheme for *Coxiella burnetii*. *Federation of European Microbiological Societies*, 268-274.

Tilburg, J. J., Melchers, W. J., Pettersson, A. M., Rossen, J. W., Hermans, M. H., Hannen, E. J., Nabuurs-Franssen, M. H., Vries, M. C., & Horrevorts, A. M. (2010). Interlaboratory evaluation of different extraction and Real-time PCR methods for detection of *Coxiella burnetii* DNA in serum. *Journal of Clinical Microbiology*, 3923-3927.

Tilburg, J. J., Rossen, J. W., van Hannen, E. J., Melchers, W. J., Hermans, M. H., van de Bovenkamp, J., Roest, H. J. I. J., de Bruin, A., Nabuurs-Franssen, M. H., Horrevorts, A. M., & Klaassen, C. H. W. (2012). Genotypic diversity of *Coxiella burnetii* in the 2007-2010 Q fever outbreak episodes in The Netherlands. *Journal of clinical Microbiology*, 1076-1078.

Vaidya, V., Malik, S., Kaur, S., Kumar, S., & Barbuddhe, S. (2008). Comparison of PCR, immunofluorescence assay and pathogen isolation for diagnosis of Q fever in Humans with spontaneous abortions. *Journal of clinical Microbiology*, 2038-2044.

Van Schaik, E. J., Chen, C., Mertens, K., Weber, M. M., & Samuel, J. E. (2013). Molecular pathogenesis of the obligate intracellular bacterium *Coxiella burnetii*. *Nature Reviwes Microbiology*, 561-573.

Vergnaud, G., & Pourcel, C. (2006). Multiple locus VNTR (variable number of tandem repeat) analysis. In G. Vergnaud, & C. Pourcel, *Molecular Identification, systematics and Population Structure of Prokaryotes* (pp. 83-104). Paris: E. Stackebrandt.

Zhang, G. Q., Hotta, A., Mizutani, M., Ho, T., Yamaguchi, T., Fukushi, H., & Hirai, K. (1998). Direct identification of *Coxiella burnetii* plasmids in Human sera by Nested PCR. *Journal of Clinical Microbiology*, 2210-2213.

7. CYBERGRAPHIC REFERENCES

[RC1] - http://www.wormsandgermsblog.com (access date: August 2013).

[RC2] - http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.12_Q-FEVER.pdf (access date: August 2013).

[CR3] - http://www.bio.davidson.edu/courses/genomics/method/nestedpcr.html (access date: August 2013).

[CR4] - http://www.ebiotrade.com (access date: June 2013).

[CR5] - http://www.wvdl.wise.edu/PDF/WVDL.Info.PCR_Ct_Values.pdfs (access date: June 2013).

[CR6] - http://mlva.u-psud.fr (access date: August 2013).

[CR7] - http://minisatellites.u-psud.fr/MLVAnet/ (access date: August 2013).

[CR8] - http://mlva.eu/ (access date: August 2013).

[CR9] - http://www.umcutrecht.nl/subsite/MLVA/ (access date: August 2013).

[CR10] - http://miru-vntrplus.org/ (access date: September 2013).

[CR11] - http://www.pasteur.fr/recherche/genopole/PF8/mlva/ (access date: September 2013).

[CR12] - http://mlva.net/ (access date: September 2013).

[CR13] - http://insilico.ehu.es (access date: September 2013).

APPENDIX

Parts of the complete genome sequence of the reference strain Nine Mile RSA 493 available on GenBank (accession nr. NC_002971), in which are signed each MLVA locus nucleotides (forward and reverse) and the repeat motifs.

TGATTTACAGGAACGATTATTAGCGGCAATAGCTAATCTTAATGATGAGAGAAACGAGAGA
GAAGTAAGAGGAAGGGCTGGAAGGGGTGCCAGAAGAAGATCCGGAGCACCCGTTATTGA
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CTTAAAGTAATATCCAAAAATTAATTCAATCTTA**ACGGGCTAAATTACACCTGCT**TTTTTGGA
CAAAAATCAATAGCCCGTATGGAGGGCA/GAAGACA/GAGGACA/GAGGACA/GAGGACA/GA
GGACA/GAAGACA/GAAGACA/GAGGACA**GTGGTTGTGTCGCTATGCG**ACAACTAAATTTTA
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GATAAGTC

Ms23_F →

← Ms23_R

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Ms24_F

Ms24_R


Ms27_F
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AAGTCGAATCGCCCGCTTGTCGAATGCGTTGCGCTAAGAAATACGCACCAAACCGTTCGC
GTTCTTGAATGGCTGCTAGGGTATCCTGCCATTGTGCGGTTTCACGAAAGCTGAAATAGAG
TTTCACAAGCATAATCATTAATAAAGAGCCTAAAAACAGAGACACCAATAATTCAATTAGACT
GAACCCTGTTTGCAATTTCATACCGCTGCAACCAAAGATAAAGTTTGTATTTTTCTCTCCCG
CCAATGAACGGTCACGTGGCAAATACGTCGTTCACAGCGATAATTCCCTTCTCCTTGCGGC
AACACCCCCTTATTCTCATTATTCCAATCCCTCCATTCCCGTTCACGCGCAACGGAAGTTTG
ATTCGCGCGCAGTCGTTCTATTAAAGATCGTAATTGAACCGTAGCTACGCTGTAATAATAAG
CATGACGAATATGGCGAACCTCATCGATTTGTAAAAACAAAACACTTAATAAAATACCGGAT
AAAATAGACCAGGCAATTAATACCTCAAGCAATGTAAACCCGTAAGAAGGTGAATGACTCA
TCGAGAAAGAGTAAAAAGGAAAAATAAATGAGTAAATACCGTGATTCCCTAGGCAGAGGAC
AGAGGACAGTGTTCGTGTGGCTTCGCCACAATTAATTTATAAAATTTAGTTG**TCGCGTAGC**
Ms33_F
GACACAACCACTGTCTT/CTGTCTT/CTGTCTT/CTGCCCTCTGTCTT/CTGCCCT/CTGCCCT/
CTGCCCT/CTGCCTTCC**GTTCGCGTCATACGGGCTA**CATAATTAATCACTTACTTTCTTTTC
Ms33_R
GAAAATTTTTAAAATATCGATTGGCATGGGGAAGACAATGGTTGAAGTTTTATCGCT

Ms34_R

CCATAACCATTCTCCTAGGTAAGGCGTACGAAGAATCTAAGTGCTGGAA**CAATGACTATCA**

GCGACTCGAAGAAAAAAGCACAGACCTTGAAAAG/GAAAAG/GAAAAG/GAAAAG/GAAAAG

GAAAAATTAA**CACAGCAACTCACCGAAGAA**CAGGAGCGTTCTCGTACTTTAAGTGAAAAA

Ms34_F

GTCACCCTTTTAAATCAGGAATTGAAAAACCTTGAGCAAGTAATTAACCAAAAGAATCAAGA

GATAAGCACACTAACGCACGAAAATTCACAGTTTCAGCGTCTAGAAAAAGAAAACTCGACA

CTCACCGAAAAAATTATATATTTACAAAAGATTGTGAGGCTTGCGATGCAATTTCAGGAGGA

GCCAAAAACAGTTGCGTGTTCAAACGAAGCCATTGCGCCTCCTCTCGCCATGCCATTTTCG

TCTCCTGTTTCACCTCTCGATGGTTCTCTTATTCCTTCCGAAGAAAAGGAAGATTCTTCACC

TAAAAGAACTCGTGTCGACCACTCTTCTTCGTCAACTTTTTTAGGGTCATTCTTTAAATCAG

CTGAATCTAGCAACAAGGAGGAGGGCTACGATCACGCTCCCAAAAGGACATAGGGATTTC

ACAAAATTGAATTTTTAACCCTCCGCAAGCAGGGAAACAAAGCGTCTTAAATCGTAAGGCG

CTTTGTTTCTTCCGGGTCGTCTGGCTGCTTTTCGTCTTCCCAATTAGGGATGGCCGCTTTTT

CTTCGGGTGAAAATATTTGTTGCCGGATTATTTTTTTCGCATCTGGATTTTTCAATAATTCTT

GGATGATATTTTTTCCATCCTCCCATTGAGTCTTCAGGAGCCAGCGGAATGCTTCTGGACC

AAGGAGTGTTAATATAAGATCAAGGCTATTTCTTTTTTTTCTGAGTAGTGTCTTTTGAATGCA

TTCCCGAATGTAAGAATTTTTTGGAAGTCGATTTAATAGTCCGCTTTTAGACTTAAAAAGAAA

TTCGACTCCTCCGGCTTCTTCGCAGATATCGCAAGCGGGTAGTATTAAGGATTGAGGGATT

GTTCGATCGAATAATTGACGTACAATGCGGTTCTGCAATTCATTAACTATCGCAGGATTTAC

CCACGAAGCCCCAATACCCAAATAACTAAAAAATCCCAGTGCGTGATCATCCGAACATAGC

AGCATCCAGAAAGGTAATCCAAAACGGCTACAGGTGTAAAATTGTTTTAAAAAACGACGATT

GACATGATAATCAATAACGGGCAGCAGGCCCTCCTTTCGGCTATCGTATAGTAACGTTCTC

CCTCCTTCCGATAAGCATAAACCAAACAAATTCTTTTTAAGAAATATGGAAATCATATCGGTA

ACTGAGAAGCTCTCGTGGCCTATAGAGTAGAAAAGCGCCTCTAATTCCAGGGCAGTATTGG

AGTTTCGACACAGCCATGCCCAAGCGGGGACTTTGACTTTGCCAACGAGTCCTGTTTGATA

CAAGTGATGACTCAATAAACTCTTAAGTTCTACATCGGGAAACGAACTCTTGAAATAACGAA

GTTGTCTGAGACGGGTTAGTAATTTGGGTGTGTGGCAACACTCTCTGTCAGCTCCGCATAA

GCAATAAACAAAAGAGTAATCAACTGAACTGAGCCGCCTAATACTCCACAGCGCCAATAAT

TTTTTAAAATGGTCTGGGCTAACTGTAGCCAGTAGATAATCGATAGTGTTGCGTAATCCATA

ACCTCCGATCGAAAGTGGAACCTGGCTTGTAAATAACGATTTGACCAAGTCGTATCCTTCTT

CCGAAGTAAAATCGCTGATCAAGATATCGCACAGGTGATTAATTGCTCCTGTTGCGATTAAA

AGAGATAGCGTTGTCGTATTCGCAAGATTTCCAGCACCTTTATCTATCGGTTGACATAAACC

AGAAAAACAATCCCTGATGTCAGTGTGCAGCCCCCGTATATCATTCTACGAATGAAAGAATT

GAAAAAATCAGCAGCATGTCCGTGTGCGTGGAAAATTAAAAAATTAAAAATACTCGGCAAAT

Table 1 - *Coxiella burnetii* table for alleles assignment [to convert alleles size (bp) into number of repeats (U)]

	Nr. of repeats / Alleles sizes (bp)																										
Locus	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**
Cbu0197_ms23_7bp_9U_133bp	84	91	98	105	112	119	126	133	140	147	154	161	168	175	182	189	196	203	210	217	224	231	238	245	252	259	266
Cbu0259_ms24_7bp_27U_261bp	79	86	93	100	107	114	121	128	135	142	149	156	163	170	177	184	191	198	205	212	219	226	233	240	247	254	261
Cbu0838_ms27_6bp_4U_89bp	71	77	83	89	95	101	107	113	119	125	131	137	143	149	155	161	167	173	179	185	191	197	203	209	215	221	227
Cbu0839_ms28_6bp_6U_111bp	81	87	93	99	105	111	117	123	129	135	141	147	153	159	165	171	177	183	189	195	201	207	213	219	225	231	237
Cbu1435_ms33_7bp_4U_104bp	48	55	62	69	76	83	90	97	104	111	118	125	132	139	146	153	160	167	174	181	188	195	202	209	216	223	230
Cbu1471_ms34_6bp_5U_101bp	77	83	89	95	101	107	113	119	125	131	137	143	149	155	161	167	173	179	185	191	197	203	209	215	221	227	233

*Yellow boxes – expected size (bp) in *C. burnetii* Nine Mile RSA493